University of Alberta

Regulation of Phosphatidate Phosphatase-1 (Lipin) Expression in Rat and Mouse Hepatocytes by Glucocorticoids, cAMP and Insulin

by

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TO MY FAMILY

ABSTRACT

Phosphatidate phosphatase-1 (PAP1) is an enzyme essential for the synthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine. Although much is known about PAP1, the protein(s) responsible for PAP1 activity were not identified until 2006 when yeast PAP1 was shown to be the orthologue of mammalian lipin-1, -2, and -3. My work used primary cultures of mouse and rat hepatocytes to study the hormonal regulation of the expression of lipin-1, -2 and -3. Here I reported an increase in lipin-1 mRNA, and protein together with PAP1 activity induced by dexamethasone, a synthetic glucocorticoid. The inductions were synergized by CPTcAMP and antagonized by insulin. We conclude that lipin-1 is responsible for the inducible PAP1 activity that results in increased TAG synthesis, causing steatosis found in many conditions such as starvation, diabetes, and toxic conditions in the liver.

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LIST OF ABBREVIATIONS

ActD	Actinomycin D
AGPAT	acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase
bp	base pair
BSA	bovine serum albumin
CH	cycloheximide
CPTcAMP	8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphate
CT	CTP:cholinephosphate cytidylyltransferase
DAG	diacylglycerol
dex	dexamethasone
DGAT	diacylglycerol:acyl-CoA acyltransferase
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
FA	fatty acids
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoids
glu	glucagon
GPAT	Glycerol-*sn*-3-phosphate acyltransferase
HBBS	Hank's balanced salt solution
HBS	HEPES buffered saline
IP	immunoprecipitation

Kb	kilo base pairs
LPP	Lipid phosphate phosphatase
LPA	lysophosphatidate
mRNA	messenger ribonucleic acid
NEM	N-ethylmaleimide
OD	optical density
PA	phosphatidic acid
PAP1	phosphatidate phosphatase 1
PAP2	phosphatidate phosphatase 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC	phosphatidylcholine
PE	phosphatidylethanolamine
p/s	penicillin-streptomycin
RNA	ribonucleic acid
Rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SSII	superscript II reverse-transcriptase
TAG	triacylglycerol
TE	tris-EDTA

MATERIALS

REAGENTS:

10 bp DNA Ladder	Invitrogen
100 bp Plus DNA Ladder	Invitrogen
Actinomycin D (ActD)	Sigma
Agarose	Gibco
BSA	Sigma
Calcium Chloride ($CaCl_2$)	Fisher
Collagenase	Sigma-Aldrich
Cycloheximide	Sigma
CPTcAMP	Sigma-Aldrich
D-glucose	Sigma
dNTPs	Invitrogen
Dexamethasone	Sigma-Aldrich
Diethyl ether	Caledon
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Invitrogen, Fisher Scientific
DNA-free	Ambion
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen
Ethanol	BD Biosciences
Ethylenediaminetetraacetic acid (EDTA)	Fisher
Ethyleneglycoltetraacetic acid (EGTA)	Sigma
Euthanyl	Vetoquinol N.-A. Inc
Fetal Bovine Serum (FBS)	Gibco
Glucagon	Sigma-Aldrich
Glycerol	Anachemia
Hank's balanced salt solution (10x) (14185-052)	Gibco
Hank's balanced salt solution (10x) (14065-056)	Gibco
HEPES	Fisher Scientific
Insulin	Sigma

Magnesium Chloride ($MgCl_2$)	Sigma
Methanol	BD Biosciences
Metofane	Janssen
NEM	Sigma
Nuclease-free water	Gibco
Penicillin-Streptomycin	Gibco
Protease Inhibitor Cocktail	Sigma
Random Primers	Invitrogen
RNAqueous	Ambion
RNAseOUT	Invitrogen
Saline	Baxter Corporation
Cytoscint	MP Biomedicals
Silica	Fisher
Sodium Dodecyl Sulfate (SDS)	Schwarz/Mann
SSII	Invitrogen
SYBR Green Mastermix	Applied Biosystems
Tris	Sigma
TritonX-100	Sigma
Tween-20	Sigma

CHAPTER 1

INTRODUCTION

1.1. Introduction

Obesity and the Metabolic Syndrome are becoming more prevalent in many countries around the world. These conditions are strongly associated with an increased risk of atherosclerosis, coronary thrombosis and stroke. Therefore, it is very important to understand the changes in hormonal and metabolic balance which govern these symptoms. For over two decades the Brindley lab has studied the effects of insulin and other counter-regulatory hormones such as glucocorticoids or glucagon, as well as fatty acids on the development of obesity, insulin resistance and metabolic syndrome [1-6]. It has been shown that an imbalance of the various hormones can result in deleterious effects. For example, glucocorticoids cause insulin resistance and increase the lipolytic effects of growth hormone and catecholamines [7]. Excessive lipolysis in adipose tissue results in an increased fatty acid load to the liver [8]. This in turn drives triacylglycerol (TAG) synthesis and the secretion of very low-density lipoprotein (VLDL) [4]. The Brindley lab has shown that the increase of TAG synthesis was achieved mainly through the increase in phosphatidate phosphatase (PAP1) activity [2, 9] , which is catalyzed by a family of lipin proteins [10]. The lipins are the main focus of this study.

1.2. TAG synthesis pathway

PAP1 is involved in penultimate step of TAG synthesis (Fig 1.2). In the TAG synthesis pathway, glycerol-3-phosphate is acylated with acyl-CoA yielding LPA. The reaction in this step is catalyzed by an enzyme called acyl-CoA:Glycerol-*sn*-3-phosphate acyltransferase or GPAT [11, 12]. The glycerol-3-phosphate substrate is generated by the reduction of dihydroxyacetone-3-phosphate by dihydroxyacetone-3-phosphate

dehydrogenase or by the phosphorylation of glycerol by glycerokinase. The acyl-CoA substrate is produced by one of several long-chain or very-long-chain acyl-CoA synthetases [13, 14]. There are two isozymes of GPAT identified namely microsomal and mitochondrial GPAT [15].

In the next step, LPA is acylated in the reaction catalyzed by acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase or AGPAT (also known as lysophosphatidate acyltransferase or LPAAT) [16]. The product obtained from acylation is PA, which is the substrate for PAP1 as well as PAP2. AGPAT activity is located in both mitochondria and microsomes [16]. The enzyme activity has been detected in the plasma membrane [16].

Following the synthesis of PA, DAG is produced from a removal of PA's phosphate group catalyzed by phosphatidate phosphatases (PAPs) [17]. There are two types of PAP found in mammalian tissue namely PAP1 and PAP2. PAP1 is the main focus of this study and will be discussed in detail afterwards.

Diacylglycerol lies at the branch point between PC, PE, and TAG synthesis [18]. In PC production, choline is phosphorylated in ATP-dependent reaction [19]. Subsequently, CTP:cholinephosphate cytidylyltransferase (CT) [19] catalyzes the formation of activated intermediate CDT-choline [19]. In the final step, choline phosphotransferase mediates the transfer of choline phosphate moiety of CDP choline to DAG yielding PC [19]. There are

two isoforms of CT namely CTα and CTβ. CTα is predominately found in the nucleus whereas CTβ is cytoplasmic [19].

DAG also has another fate. In the final step of TAG synthesis pathway, DAG is converted to TAG by enzymes called diacylglycerol:acyl-CoA acyltransferase (DGAT) 1 and 2 [20]. TAG produced becomes available for storage in a form of cytosolic droplets or for VLDL secretion.

1.3 The Phosphatidate Phosphatases (PAPs)

The phosphatidate phosphatases are classified into phosphatidate phosphatase-1 (PAP1) and phosphatidate phosphatases-2 (PAP2), the latter of which is also known as lipid phosphate phosphatases (LPPs).

The phosphatidate phosphatase-1 (PAP1, 3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) enzyme catalyzes the dephosphorylation of phosphatidate (PA), yielding diacylglycerol (DAG) and inorganic phosphate (Figure 1.1). PAP1 has a principal role in the synthesis of phospholipids and TAG through its product DAG. In addition, PAP1 generates and/or degrades lipid-signaling molecules such as DAG, for the activation of protein kinase C, and it degrades PA, which is also bioactive [21]. The PAP1 reaction is the committed step in TAG synthesis (Figure 1.2). DAG, the product obtained from the reaction, is used for both the production of TAG as well as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [22-24].

PAP1 activity is localized in the cytosol. It transiently interacts with the endoplasmic reticulum at the cytosolic surface, where glycerolipid synthesis occurs [25]. Mammalian phosphatidate phosphatase (PAP1) activity is Mg^{2+}-dependent and is inhibited by *N*-ethylmaleimide (NEM) [17]. These characteristics distinguish mammalian PAP1 from PAP2 activities that also convert PA to DAG. PAP2 is now commonly known as a family of lipid phosphate phosphatases (LPPs) that dephosphorylate a variety of lipid phosphate esters. The LPPs are mainly involved in regulating signal transduction by bioactive lipid phosphates such as PA, lysophosphatidate (LPA), sphingosine 1-phosphate and ceramide 1-phosphate [26-29]. The catalytic sites of the LPPs are localized to the external surface of the plasma membrane or the luminal surface of internal membranes [27]. By contrast, PA appears to be a specific substrate for PAP1 [10, 30] which is a required enzyme in the biosynthesis of TAG, PC, and PE [31].

Unlike the LPPs, which do not have a Mg^{2+} requirement, PAP1 requires Mg^{2+} to function [17, 32]. The requirement of the cofactor Mg^{2+} ion is related to the catalytic motif DxDxT within a haloacid dehalogenase (HAD)-like domain. The DxDxT motif is found in a superfamily of Mg^{2+}-dependent phosphatase enzymes, and its first aspartate residue binds the phosphate moiety in the phosphatase reaction [33, 34].

The following table summarizes the similarities and differences between the two members of the PAP family, PAP1 and PAP2 (LPPs).

Table 1.1 Summary of major characteristics and properties of PAP1 and PAP2

Characteristics	PAP1	PAP2 (LPPs)
Mg^{2+}	Dependent	Independent
NEM	Sensitive	Insensitive
Temperature Sensitivity	> 30 °C	> 50 °C
Localization	Cytosol; translocates to ER or nucleus	Plasma Membrane
Specification	PA	Non-specific
	Catalyzes dephosphorylation of PA to DAG and Pi	Catalyzes dephosphorylation of bioactive lipid phosphates

1.4. Long Term Regulation of PAP1 Activity

Previous studies by the Brindley group showed that injecting rats with cortisol or corticotrophin produced marked increases in PAP1 activity in the liver [35, 36]. Subsequent work with rat hepatocytes demonstrated that the glucocorticoid (GC) effect in increasing PAP1 activity was synergized by glucagon and inhibited by insulin [1, 37]. It has been shown that these GC-induced increases in PAP1 activity provide the extra capacity for the liver to sequester excess FAs as TAG when these FAs are not immediately required for β-oxidation [31]. The interplay of effects of GC and insulin explains the diurnal rhythm of PAP1 activity in rat livers [38]. The GC effect is also consistent with the increases in hepatic PAP1 after sham operations, in liver remnants after partial hepatectomy [6], starvation [35], diabetes [39], insulin resistance [40], hypoxia [41], and toxic conditions [31]. Increases in hepatic PAP1 activity also occur in response to dietary modification in rodents, for instance, when glucose or starch is replaced by fructose, sorbitol, glycerol, or ethanol [5], and these effects are exaggerated by high-fat feeding [42]. These dietary-induced changes in PAP1 are also associated with

increased GC concentrations relative to insulin. PAP1 activity is also increased in the livers of alcoholic baboons [43] and human [44]. The involvement of GC in ethanol-induced increases in PAP1 activity is confirmed because this is attenuated in adrenalectomized rats [45].

Previous studies using primary cultures of rat hepatocytes have revealed a dramatic increase in PAP1 activity when the glucocorticoid dexamethasone (dex) was added to the culture medium. A synergistic effect was also observed with glucagon (glu) or cAMP. The increases in PAP1 activity induced by dexamethasone and glucagon were attenuated by insulin [1, 37]. Furthermore, glucagon increases the half-life of PAP1 activity by about 2-fold whereas insulin decreases it [46].

1.5. Short Term Regulation of PAP1 Activity

PAP1 activity is primarily found in the cytosol and can translocate to the ER membrane to gain access to its substrate, PA. PAP1 translocates to membranes of rat hepatocytes in response to FAs or acyl-CoA [47, 48]. Cytosolic PAP1 acts as a reservoir of activity that interacts with the ER or mitochondria to access newly synthesized PA [31]. This control mechanism for the translocation of the enzyme inducing PAP1 activity to the ER provides reserve capacity, which allows the liver to respond to almost any FA load and sequester excess FA as TAG [31]. The sequestration of FA into TAG results in steatosis in severe diabetes, stress reactions and in toxic conditions (including alcohol intoxication).

It was previously shown that PAP1 translocation to the ER depends on the FA and acyl-coA esters binding to the ER membranes [25]. The FA, acyl-CoA esters and PA, which accumulate as a result of increased FA supply, are anionic amphiphiles. These species provide a negative charge to the membrane and a feed-forward signal for TAG synthesis by PAP1 [25]. This finding is consistent with the so-called "Nuclear Localization Signal (NLS)" domains of the lipins, the proteins responsible for PAP1 activity (Fig. 1.3), which are required for FA-induced translocation. Because the NLS domains are composed of stretches of positively charged arginines and lysines, it also explains the observation that cationic amphiphiles (e.g., chlorpromazine) displace PAP1 from the ER and overcome the FA-induced translocation of PAP1 activity [49, 50].

In recent studies, one research group treated adipocytes with insulin and found that it resulted in a reduction of electrophoretic mobility as well as an increase the phosphorylation of Ser and Thr residues in lipin [51]. The same group also reported a marked increase in the ratio of soluble to microsomal lipin-1 [52]. The finding that insulin removes lipin-1 from the membrane compartments contradicts the fact that insulin stimulates the synthesis of TAG in adipocytes.

In contrast to phosphorylation by insulin, lipin-1 is dephosphorylated by the action of epinephrine and oleic acid [52]. It has been reported that PAP1 activity was not affected by the hormone treatments by insulin or epinephrine, or by dephosphorylation of lipin-1 with protein phosphatase 1 [52]. The results suggest that insulin controls lipin-1 by

changing its localization rather than altering intrinsic PAP activity as measured with artificial substrates [52].

As pertaining to my study, PAP1 activity in the liver is increased in response to elevated conditions where glucocorticoids are elevated relative to the insulin level. The increase in hepatic PAP1 activity is commonly found in diabetes, hypoxia, and fatty liver [31].

To conclude, the physiological expression of PAP1 activity involves a FA-induced translocation of the reservoir of cytosolic PAP1 to become functional on membranes of the endoplasmic reticulum (ER), where PA is synthesized (Fig. 1.4) [48, 50]. The activity of the membrane-bound PAP1 correlates closely with the conversion of PA to DAG and the synthesis of TAG and PC in intact rat hepatocytes [50].

1.6. Identification of Lipin as PAP1 enzyme

In 2006, Han et al. sequenced a protein in yeast, which was previously known as SMP2 [30]. The gene for SMP2 encodes a protein of approximately 91 kDa, which has a molecular function of phosphatidate phosphatase [30]. It was later named as PAH1 for phosphatidic acid phosphohydrolase. The protein contains a conserved haloacid dehalogenase (HAD)-like domain in the middle of the sequence, which contains a DXDXT motif found in a superfamily of Mg^{2+}-dependent phosphatase and phosphotransferase enzymes (Figure 1.3). The authors provided evidence that PAH1 is

Mg^{2+}-dependent and insensitive to NEM [30] and that it is an ortholog of mammalian lipin which was identified previously.

The identification of mammalian lipin began when Peterfy et al. found the fatty liver dystrophy or *fld* locus and identified a gene of unknown function in this region [53]. *Fld* is the gene whose mutation is responsible for lipodystrophy in fatty liver dystrophy mouse. They reported that this gene is expressed in the liver of wild-type animals and is absent from the *fld* animals. They proposed it as a candidate gene for the *fld* phenotype [53]. They later named the gene responsible for the phenotype as *Lpin1* and the encoding protein as lipin-1 [54]. Lipin-1 is found to be required for the normal development of adipose tissue [55]. This finding allows further PAP1 study, which had been hampered by the lack of a known gene responsible for its activity

Further study revealed that besides PAH1 in yeast and lipin-1, -2, and -3 in mammals, a single lipin gene ortholog is also found in other organisms. The gene was found in nematode, fruit fly, fish and plants [54].

1.7. The Lipins

1.7.1 Overview of the lipins

There are three mammalian members in the lipin family, which consists of lipin-1, -2 and -3. (Fig.1.4). Lipin-1 has two splice variants named lipin-1A and lipin-1B. Lipin-1A, which has a predicted size of approximately 98 kDa, is found predominantly in the nucleus; lipin-1B, whose size is approximately 102 kDa, is found primarily in the

cytoplasm [56]. Through sequence similarity analyses, the other two members of the lipin family, lipin-2 and lipin-3, were discovered [54]. These latter members of the lipin family share approximately 44-48% amino acid identity with lipin-1 [54].

The three members of the lipin-1 family share some similarity as well as distinct properties. They share functional motifs such as the N- and C-terminal domains and the HAD-like domain, and all have PAP1 activity. However, they exhibit distinct tissue distribution and distinct diseases associated with mutated genes. Interestingly, Lipin-1A and lipin-1B share similarity to CTα and CTβ, respectively in terms of their localization.

1.7.2 Structure, tissue distribution, localization of the lipins and PAP1 activity

Donkor et al. studied the three members in the lipin family (Fig 1.5) and found that all possess PAP1 activity [10]. They were found to be Mg^{2+}-dependent and have distinct tissue expression patterns. Lipin-1 is found predominantly in skeletal muscle, with lower levels in adipose tissue, brain, and liver. Lipin-2 is found in liver and brain, while lipin-3 has significant expression in intestine and other regions of gastrointestinal tract such as liver. The authors suggest that each lipin may perform similar biochemical functions but may act in a tissue-specific manner [10].

1.7.3 Biological functions of lipins

Lipin-1 is required for the development of mature adipocytes [57, 58]. Studies carried out in adipose tissue obtained from the *fld* mice as well as lipin-1 deficient cells revealed significant defects in the induction of genes relevant to the adipogenic program. This

involves peroxisome proliferator-activated receptor γ (PPARγ) and CAAT enhancer binding protein-α (C/EBPα), as well as their downstream target genes [55]. In contrast to lipodystrophy which is caused by lipin-1 deficiency, overexpression of lipin-1 promotes obesity. On a high-fat diet, lipin-1 transgenic mice gained weight at a faster rate than non-transgenic mice, despite similar amount of food intake [59].

The expression of hepatic lipin-1 is promoted by PPARγ coactivator 1α (PGC-1α). In turn, lipin-1 activates a subset of PGC-1α target pathways including β-oxidation and it inhibits FA synthesis [60]. This is achieved through the formation of the lipin-1/ PPARα/ PGC-1α complex, which amplifies the control of hepatic metabolism by PGC-1α and PPARγ. The result is an activation of FA oxidation genes during fasting [60].

Lipin-1 levels in adipose tissue have been reported to increase insulin sensitivity and reduced inflammatory cytokine expression in humans [61]. It has also been reported that there was a negative correlation between lipin-1 expression levels and glucose metabolism as seen in fasting glucose levels, insulin levels and insulin resistance [59]. By contrast, in muscle, an enhanced lipin-1 expression results in obesity-relate to insulin resistance [59]. A study has revealed that muscle-specific lipin-1 transgenic mice had increased feed conversion efficiency and reduced oxygen consumption and energy expenditure [59]. The muscle-specific lipin-1 transgenic mice also had increased fasting glucose levels when they were fed a high-fat diet and increased plasma insulin levels

when they were fed either a regular chow or a high-fat diet [59]. This implicates lipin-1 as one of the determinants in glucose homeostasis [61].

In a comparison of the diurnal metabolic fuel switching in wild-type mice and *fld* mice, it has been reported that the *fld* mice exhibited abnormal fuel utilization throughout the diurnal cycle [62]. The authors found that the inability of *fld* mice to store energy in adipose tissue resulted in a compensatory increase in glycogen storage for use during fasting and reliance upon hepatic FA synthesis to provide fuel for peripheral tissues during a fed state [62]. They suggested a role for lipin in the coordination of peripheral glucose and fatty acid storage and utilization [62].

Lipin-2 had been evaluated as a candidate gene responsible for an occurrence of high-grade myopia or nearsightedness but this possibility was ruled out in 2005 [63, 64]. However, in the same year, mutations in lipin-2 were reported to cause Majeed syndrome, which is characterized by chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia [65-67]. Since Majeed syndrome is an autosomal recessive, autoinflammatory disorder, the study was carried out among Jordanians who have a high prevalence of consanguinity. Three cases were reported with varying severity. The authors speculated that this was probably due to different mutations resulting in amino acid change and altered structure and function of the protein versus truncated protein resulting in loss of function [65, 66]. The counterpart gene to lipin-2 identified in mouse is called *pstpip2*, whose mutation results in chronic multifocal

osteomyelitis (cmo) [67, 68]. The cmo mice develop tail kinks and hind foot deformities caused by osteomyelytis in the affected bones [68].

In addition to Majeed syndrome, a couple of research articles published reported that lipin-2 is associated with insulin sensitivity, body mass index (BMI) and type-2 diabetes [69, 70]. Unfortunately, the mechanisms by which lipin-2 causes the aforementioned symptoms remain unknown. Currently, there is no information regarding the function of lipin-3.

1.8. Hypothesis and Thesis objectives

For the past two decades, a great deal of research pertaining to PAP1 activity was delayed due to the inability to purify or identify the structure of the enzyme. Fortunately, the situation changed when Han et al. [30] published an article in February 2006 identifying yeast PAP1 as an ortholog of mammalian lipin.

The discovery that the liver expresses the three lipins -1A, -1B, -2 and -3 [10], provokes the question of which lipin(s) respond to hormonal regulation leading to the observed physiological changes in the composite PAP1 activity. Previous studies showed that PAP1 activity in adipose tissue decreased in starvation and diabetes [71]. Administration of insulin could restore PAP1 activity in diabetic state within 2 h [72]. Because of the short PAP1 activity restoration time, it is more likely that PAP1 activity was restored by reactivation rather than from a new synthesis. Moreover, there were other studies which reported that there was no change in PAP1 activity after 24-48 h of starvation [73] or

there was a significant decrease in PAP1 activity but the activity was very slowly restored at the beginning and then completely restored to the control value after 24-48 h of re-feeding [74]. These studies suggest that the PAP1 activity in the adipose tissue cannot be induced in a short term. Contrary to what is observed in the adipocytes, PAP1 activity in the hepatocytes increased within 6 h when the cells were treated with corticosterone and this increase was antagonized by insulin [75].

As mentioned earlier, each lipin exhibit distinct tissue expression patterns [10]. Lipin-1 is the primary PAP1 in adipose tissue and skeletal muscle whereas lipin-2 and lipin-3 are the predominant PAP1 in the brain, liver and intestines [10]. A separate article reported that the liver PAP1 activity was maintained in the *fld* mice, which lack lipin-1, but express lipin-2 and -3 [60]. Because lipin-1 is the only PAP1 in adipose tissue and previous experiments suggested that PAP1 activity in adipose tissue cannot be acutely induced, **we therefore hypothesize that lipin-2, lipin-3 or both, but not lipin-1, are the inducible lipin(s) that can undergo differential regulation in hepatocytes in response to hormones such as glucocorticoids, glucagon and insulin.**

It is important to answer the question which lipin is the inducible lipin in the liver and to describe the mechanisms that control the expression of these different lipins in response to various hormones relevant to obesity and diabetes. What is drawn from this work would help explain hormonal regulation of the different lipins and their individual function in hepatic metabolism.

In this present work, mouse and rat hepatocytes were used in a time course experiment where responses of the cells to CPTcAMP, dex, glucagon and insulin were studied. The results obtained from this study provide the first evidence for differential regulation of the activity of different lipins in the liver. They help explain how the composite changes in PAP1 (lipin) activity may coordinate increased TAG synthesis, β-oxidation, and VLDL secretion in conditions of starvation, metabolic stress, insulin resistance and diabetes.

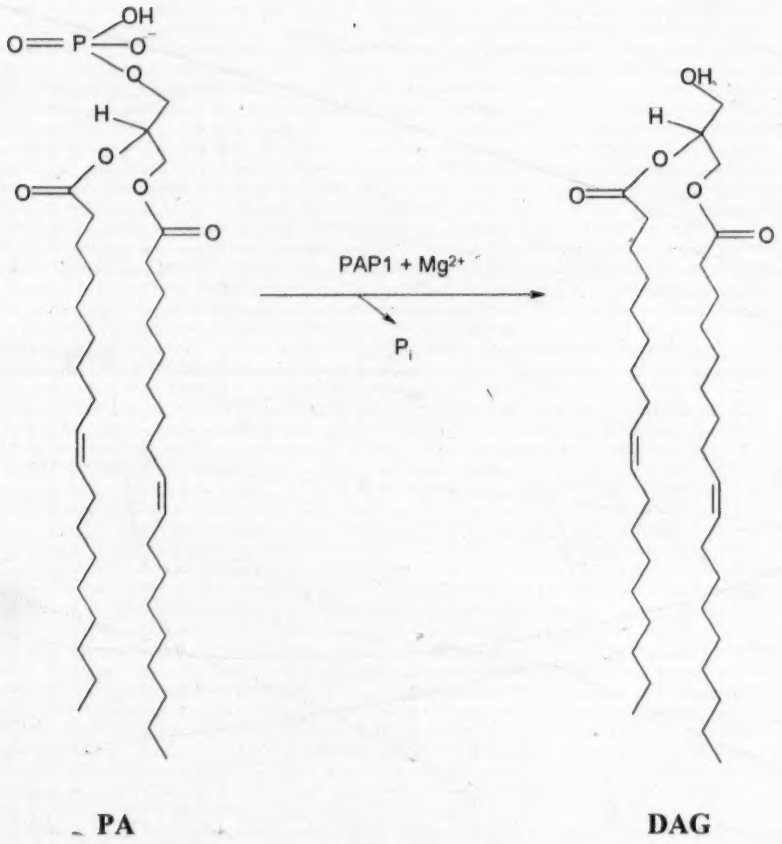

Figure 1.1 PAP1 catalyzes the removal of Pi from PA. PAP1 catalyzes the dephosphorylation of PA to yield DAG. (adapted from Carman, G. M. and Han, G., 2006) [21]

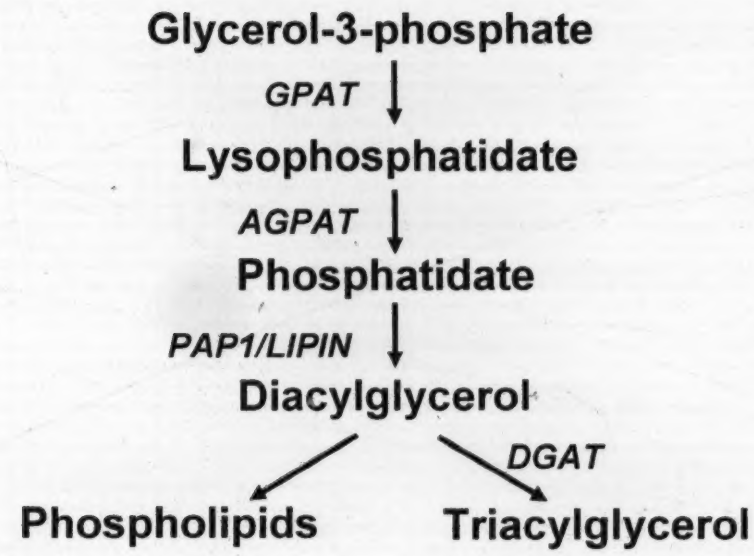

Figure 1.2 Overview of triacylglycerol synthesis pathway. Triacylglycerol (TAG) is synthesized from diacylglycerol, which could be generated by the cleavage of phosphate group from phosphatidate (PA). This penultimate step in TAG synthesis is catalyzed by phosphatidate phosphatase 1 (PAP1) or the lipins, and phosphatidate phosphatase 2 (PAP2), which is now known as lipid phosphate phosphatase (LPP) (from Coleman, R. A., 2007) [76]

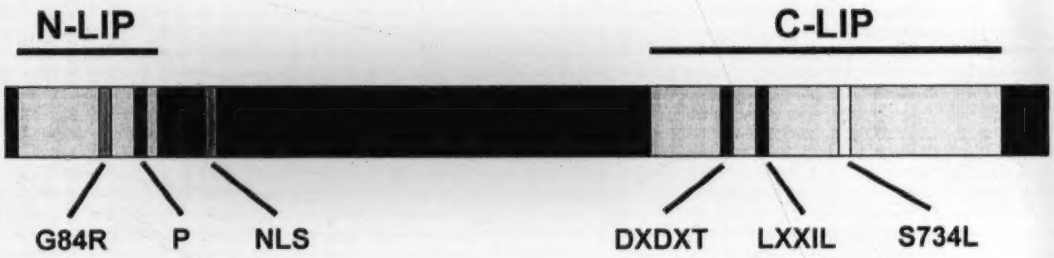

Figure 1.3 Functional motifs and disease mutations in lipin proteins. N-LIP and C-LIP domains exhibit conservation between family members and in lipin orthologs from all species. NLS, nuclear localization signal; G84R, mutation in *Lpin1* that causes lipodystrophy; P, serine residue 106, which is known to be phosphorylated in response to insulin; DXDXT, PAP1 enzyme active site; LXXIL, transcription factor interaction domain; S734L, mutation in *LPIN2* that causes Majeed syndrome. (adapted from Reue, K. and Zhang, P.) [77]

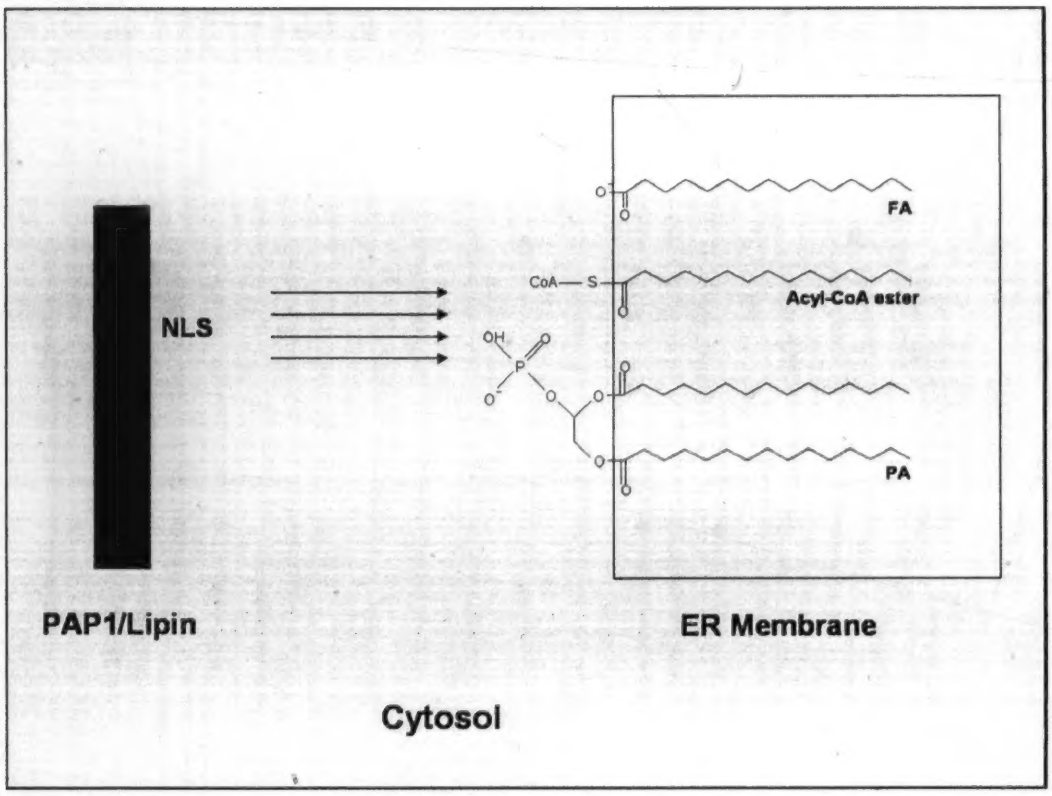

Figure 1.4 Translocation of PAP1 to ER membrane. PAP1/Lipin with a positively charged NLS region translocates to the ER membrane in response to the negatively charged fatty acids, acyl-CoA ester and PA that accumulate in the membrane [48, 50].

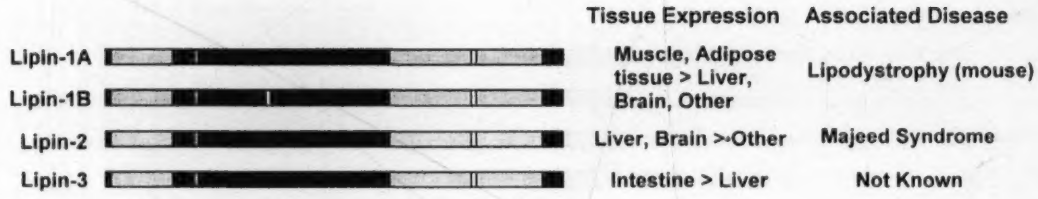

Figure 1.5 The lipin protein family. Four lipin proteins are shown, with light blue bars representing regions of highest amino acid identity, and pink line representing nuclear localization signal. Lipin-1B has a white bar representing the exon encoding additional 33 amino acids. The corresponding site of expression and associated disease are listed. (adapted from Reue, K. and Zhang, P.) [77]

CHAPTER 2

METHODS

2.1 Collagen-coated dish preparation

The bottom of 6-cm and 10-cm culture dishes was coated with a solution of 1 mg/ml type III calf-skin collagen dissolved in 0.013 M HCl. The solution was then removed. The thin film of collagen left on the dish was air-dried in the tissue culture hood for 60 min. Collagen-coated culture dishes were then rinsed once with sterile phosphate buffered saline (PBS) and twice with sterile water. The culture dishes were left partly covered to air-dry overnight. The dishes were used the following morning or stored at 4 °C for later use within seven days. The collagen coating provides a supporting mesh for attachment of the hepatocytes.

2.2 Hepatocyte perfusion

Male Sprague-Dawley rats (200 to 540 g) and C57BL/6J mice (22.5 to 32 g) were euthanized and their hepatocytes were isolated. A combination of ether, metofane and 32.5 mg/kg sodium pentobarbital injection (euthanyl) were used to anesthetize the rat or mouse to a state suitable for abdominal surgery. A reflex response test was repeatedly performed to ensure that the animal was deeply anesthetized such that it would not feel the pain caused by the procedure and was ready for the surgery.

Once the animal was in a surgical plane, a u-shaped incision was made with dissecting scissors through the abdominal skin and muscle up to the diaphragm. The tissue flap was pulled towards the animal's chest to reveal the internal organs. The intestines were gently moved to the left of the animal body to expose the hepatic portal vein.

A ligation was made and loosely tied around the hepatic portal vein close to the liver. A pair of forceps was used to pick up the top wall of the hepatic portal vein well below the ligation, and then a small cut was made approximately 1 cm below the forceps' position with microscissors. The cut was carefully made such that the opening was just big enough to allow the insertion of the round-ended needle. While the forceps were held in place, a round-ended needle was inserted into the hepatic portal vein through the cut.

While the needle was held in place, the peristalsis pump was turned on to flush HBSS with 0.5 mM EGTA through the liver. The ligature was tied tightly and the needle was repositioned so that it rested between the animal's midline and knee. The diaphragm was quickly cut and the animal's heart was immediately cut to allow the perfusate to run through. The cutting of the heart and hence exsanguination served as euthanasia. The animal's death was confirmed 10 minutes after the procedure. Once the liver turned from red to yellow, the upper vena cava was tied and the liver swelled.

The pump was stopped and HBSS with 1 mg/ml collagenase was then used to perfuse the liver. The perfusion lasted 5-10 min or until a small indentation remained for a couple seconds after the forceps tips were pressed on the liver.

The digested liver was dissected into a culture dish and the (mouse) gall bladder was removed. The liver was quickly snipped into very small pieces with a pair of scissors. The tissue was transferred to a 50-ml Falcon tube and topped with 25 mL of HBSS with collagenase and pipetted up and down to further aid digestion. All animal experiments

were performed according to guidelines established in the "Guide to the care and use of experimental animals" by the Canadian Council on Animal Care.

2.3 Primary cell culture preparation

To stop digestion by collagenase, 25 mL of complete medium which is DMEM containing 15% FBS and 100 units of penicillin and 100 ug of streptomycin/mL, was added to the hepatocytes suspension. The mixture was strained through a coarse filter (square weave wire mesh with 72 meshes per square centimeter) to remove large pieces of tissue. The filtered suspension was then centrifuged at 800 rpm for 5 min to pellet the cells. The supernatant was aspirated and the cells were resuspended in 25 mL of complete medium. The wash was repeated twice before the cells were strained through a fine filter (square weave wire mesh with 1,225 meshes per square centimeter).

A haematocytometer was used to determine the number of cells collected. Approximately 1.5 or 4.5 million cells were plated on 6-cm and 10-cm collagen-coated culture dishes, respectively. The cells were incubated in an atmosphere of 95% air and 5% CO_2 for 45 to 90 min to allow attachment. The medium was changed to fresh medium once to remove non-viable cells and the hepatocytes were incubated for a further 4 h to allow them to spread.

2.4 Treatment

The medium was aspirated and the hepatocytes were rinsed twice with Hepes Buffered Saline (HBS) prior to incubation with DMEM containing 0.1% BSA and hormone(s), or agonist(s), or a combination of the two. All incubations contained 0.5% DMSO, which

was used as a vehicle for dex. There was no significant change observed when compared the effect of control (ddH_2O) and DMSO.

The optimal concentration of the hormones and agonists used in the experiments were previously determined by Pittner et al [37] and are as follows:

CPTcAMP (cAMP)	100 μM
Dexamethasone (dex)	100 nM
Glucagon (glu)	10 nM
Insulin (ins)	100 nM
Actinomycin D (ActD)	10 μg/ml
Cycloheximide (CH)	5 μg/ml

In the experiments where actinomycin D was used, the hepatocytes were pre-incubated with the antibiotic for 30 min before dex treatment. Cycloheximide was added at the same time as dex treatment. The hepatocytes were then incubated for the specified period of time.

2.5 Cell lysate collections

2.5.1 Cell lysate collection for mRNA

The treatment medium was removed and the hepatocytes were rinsed twice with ice-cold sterile HBS. The lysis/binding solution from RNAqueous kit (Ambion) was used to collect the cell lysate, according to the manufacturer's instructions. The cell lysate was then processed immediately, or stored at -80 °C until use.

2.5.2 Cell lysate collection for protein

The treatment medium was removed and the hepatocytes were rinsed twice with ice-cold sterile HBS. The PAP1 lysis buffer, containing 250 mM sucrose, 0.15% Tween-20, 2 mM DTT and 1% Protease Inhibitor cocktail, was used to collect the cell lysate. The cell lysate was then processed immediately, or stored at -80 °C until use.

2.6 RNA extraction and reverse transcriptase reaction

Total RNA was extracted by using the RNAqueous kit (Ambion) and contaminating DNA was removed by using the DNA-free kit (Ambion), according to manufacturer's instruction. The concentration and purity of the RNA was determined by using a BioPhotometer (Eppendorf), which automatically determines the concentration (μg/ml) and the A_{260}/A_{280} ratio of the RNA samples. Reverse transcription was then performed to generate cDNA by using Random Primers, Superscript II and RNAseOUT (Invitrogen), according to manufacturer's instruction. Polymerase Chain Reaction was performed on an iCycler (Bio-Rad)

The following program was employed after the random primers were added:

65 °C	5 minutes
4 °C	Afterwards until collected for next step

The cocktail containing reagents for reverse transcriptase reaction was added and the following program was employed:

25 °C	10 minutes
42 °C	60 minutes

95 °C	5 minutes
4 °C	Afterwards until collected for next step

The cDNA obtained was stored at 4 °C until used.

2.7 Real-time RT-PCR

Unless specified as obtained from literature, the primers used for real-time RT-PCR were designed using Primer3 or PrimerExpress software and are shown in Table 2.1. The real-time RT-PCR was performed using SYBR Green PCR reagent (Applied Biosystems) on the 7500 Real time PCR system (Applied Biosystems). The products from the experiment were identified on 2% agarose gels to determine correct size. The expected size of products is shown in Table 2.2, whereas the gels showing PCR products for rat and mouse experiments were shown in Figures 2.1 and 2.2, respectively. The gels were run at 150V for 70 min. Expression of the genes of interest was normalized to the house-keeping genes cyclophilin A and GAPDH. In initial experiments, the relative changes in mRNA expressions for the lipins were essentially the same when expressed relative to either reference mRNAs. Therefore, subsequent results were expressed relative to cyclophilin A mRNA. Since there was no significant difference between the ratio of each time point within each treatment group, the ratio of the house-keeping genes, CycA to GAPDH, of each treatment group were averaged and corresponding standard deviation was determined. The result is presented in Figure 2.1 shown below.

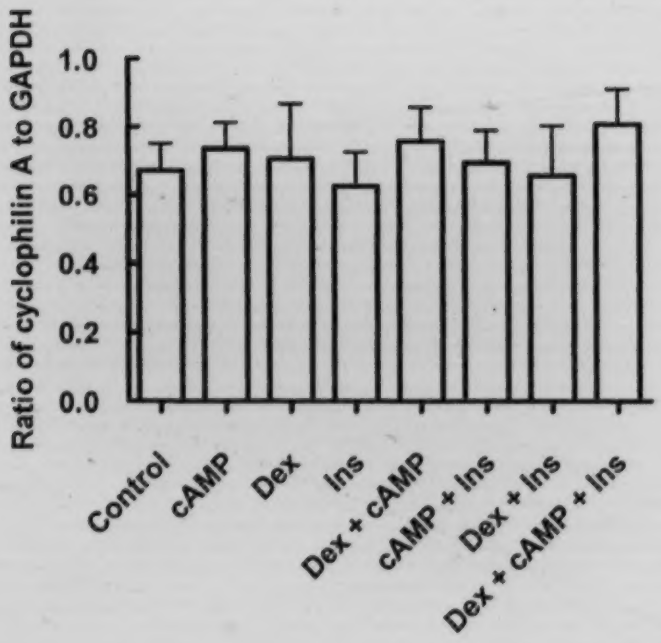

Figure 2.1 The average of the ratio of cyclophilin A to GAPDH mRNA expression of each treatment group. Results are means ± SD for 7 time points in each treatment group: A, control (0.67 ± 0.08); B, 100 μM CPTcAMP (0.74 ± 0.08); C, 100 nM dexamethasone (0.70 ± 0.16); D, 100 nM insulin (0.62 ± 0.10); E, 100 nM dexamethasone + 100 μM CPTcAMP (0.76 ± 0.10); F, 100 μM CPTcAMP + 100 nM insulin (0.69 ± 0.10); G, 100 nM dexamethasone + 100 nM insulin (0.65 ± 0.15); H, 100 μM CPTcAMP + 100 nM dexamethasone + 100 nM insulin (0.81 ± 0.10).

One-way ANOVA was performed and the result showed that there was no significant difference between the ratio of cyclophilin A to GAPDH mRNA expression among treatment groups at $P < 0.05$.

The mRNA ratio of gene product of interest to that of CycA were expressed as ratio relative to the value at time 0, which was set to be 1. The calculation method used was based on the method found in an article by Rasmussen, R. [78].

2.8 PAP1 assay

The assay measures the formation of DAG from PA labeled with [^{3}H]palmitate, which was prepared by the method of Martin et al. [79]. The PA was mixed with non-radioactive PC at 3:2 molar ration to ensure maximization of PAP1 activity relative to PAP2 activity. Both PAP1 and PAP2 can cleave the phosphate group off PA to generate DAG, yielding total PAP activity. Therefore, NEM was used to inhibit PAP1 activity in parallel assays, and this value was then used to subtract from total PAP activity to give PAP1 activity.

For the PAP1 assay, samples were assayed in 100 mM Tris/maleate buffer, pH 6.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mg/ml FA-poor bovine serum albumin, 0.6 mM PA labeled with [^{3}H]palmitate, (about 1 x 10^5 dpm/assay) and 0.4 mM phosphatidylcholine. The reaction was initiated by the addition of 20 µl of the [^{3}H]PA/PC mixture to the tubes already containing the other components of the assay as well as the cell lysate sample (enzyme) being measured. The tube was quickly submerged into a constantly shaking waterbath set at 37 °C and the reaction was allowed to proceed for 60-75 minutes. The reaction was then stopped by the addition of 2.2 mL PAP stop solution and alumina, and vortexed to ensure mixing. After centrifugation at 2,800 rpm for 5 minutes, 1 mL of organic layer was transferred to scintillation tube, and dried by immersing in 90 °C waterbath and blown with hot air from a hair-dryer. The organic residue remained was re-dissolved in 2 mL of Cytoscint solution. The scintillation tube was capped and vortexed and let sit for 8 hours before quantitation by scintillation counting. The method was based

on work done by Martin et al. [80]. Parallel incubations were performed in the presence of excess (8 mM) NEM to inhibit PAP1 and to compensate for any PAP2 (LPP) activity. The amounts of cell homogenate were adjusted such that the formation of DAG consumed <15% of the PA added. Reaction rates were measured at three different protein concentrations to ensure proportionality of the assay for each sample so that the relative rates of PAP1 activity can be calculated.

2.9 Statistical analysis

All statistical analyses were carried out at 95% confidence interval using GraphPad Prism 4 software. The significance of differences among treatments was analyzed by using a Student's T-test, Newman-Keuls post hoc test for a one-way ANOVA, or a Bonferroni test for a two-way ANOVA.

2.10 Recipe of solutions and media used in the experiment

1 mg/ml collagen solution
1 mg/ml type III calf-skin collagen
0.013 M HCl

PBS
8.1 mM Na_2HPO_4
1.1 mM KH_2HPO_4
138 mM NaCl
2.7 mM KCl
pH to 7.4

HBBS supplied with EGTA
HBBS (without calcium, magnesium or $NaHCO_3$)
19.43 mM glucose
4.17 mM $NaHCO_3$
25 mM HEPES
0.5 mM EGTA
pH to 7.4

Supplied with 1% 1 mg/ml insulin prior to use

HBBS supplied with collagenase
HBBS (without sodium bicarbonate)
19.43 mM glucose
4.17 mM $NaHCO_3$
25 mM HEPES
pH to 7.4
Supplied with 1% 1 mg/ml insulin and 1 mg/ml collagenase prior to use

Complete medium for primary hepatocyte culture
DMEM
15% FBS
100 units of penicillin and 100 ug of streptomycin/mL

Starvation medium for primary hepatocyte culture treatment
DMEM
0.1% BSA
Supplied with antibiotic, agonists or hormones specified in each treatment

HBS
50 mM HEPES
138 mM NaCl
2.7 mM KCl
pH to 7.4

PAP1 Lysis buffer
250 mM sucrose
0.15% Tween-20
2 mM DTT
1:100 Protease Inhibitor cocktail

PAP substrate
100 mM Tris/maleate buffer, pH 6.5
5 mM $MgCl_2$
2 mM dithiothreitol,
2 mg/ml FA-poor bovine serum albumin
0.6 mM PA labeled with [^{3}H]palmitate, (about 1 x 10^5 dpm/assay)
0.4 mM phosphatidylcholine.

PAP stop solution
95% Chloroform
5% Methanol
0.08% Olive oil

Table 2.1. Oligonucleotide primers used for real-time RT-PCR

Protein	Forward Primer	Reverse Primer	Reference
Cyclophilin A	caccgtgttcttcgacatcac	ccagtgctcagagctcgaaag	[81]
Mouse GAPDH	tgtgtccgtcgtggatctga	cctgcttcaccaccttcttga	
Mouse Lipin-1A	gcctgctcgtgaatcctct	cgatgcatcccgacagcgt	[56]
Mouse Lipin-1B	cagcctggtagattgccaga	gcagcctgtggcaattca	[56]
Rat Lipin-1	tcactacccagtaccagggc	tgagtccaatcctttcccag	
Rat Lipin-1B	agcagcctggtagattgtca	taaggggctggagtctttcat	
Rat and mouse Lipin-2	tagatgcagaccctgttccc	ctggtgctggcttcttttgt	
Rat and mouse Lipin-3	aaagactggacacaccaggg	tgctggatatcactcaggca	
Mouse PGC-1α	ggcacgcagccctattca	cgacacggagagttaaaggaaga	
Mouse PPARα	actacggagttcacgcatgtg	ttgtcgtacaccagcttcagc	[82]
Rat PGC-1α	cacaacgcggacagaactga	ccgcagatttacggtgcatt	
Rat PPARα	tggagtccacgcatgtgaag	cgccagctttagccgaatag	

Where not referenced, primers were designed by Primer3 or Primer Express v2.0 Software using default parameters

Table 2.2. The expected size of products from PCR reaction

Gene	Size (bp)	
	Rat	Mouse
CycA	114	114
Lipin-1	154	N/A
Lipin-1A	N/A	123
Lipin-1B	270	225
Lipin-2	116	112
Lipin-3	253	263
PPARα	63	76
PGC-1α	73	65
GAPDH	N/A	77

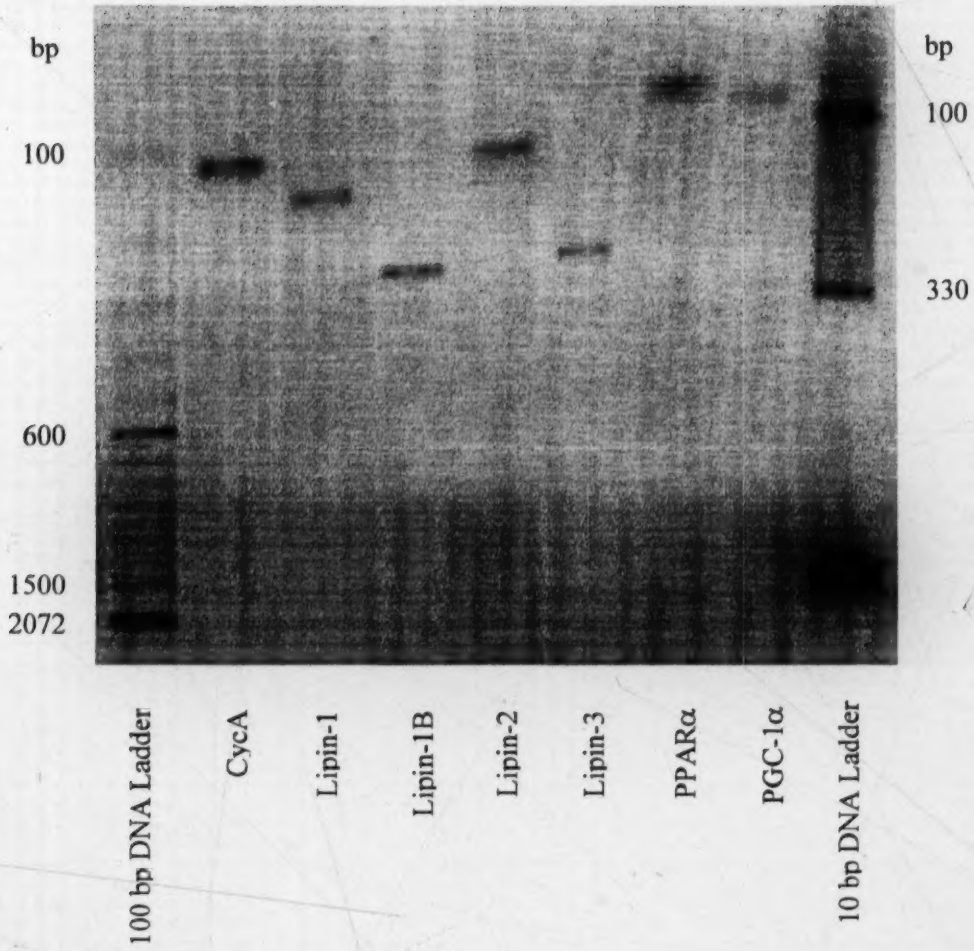

Figure 2.2. The PCR product of different rat genes of interest amplified by oligonucleotide primers for rat. The products were separated in 2% agarose gel.

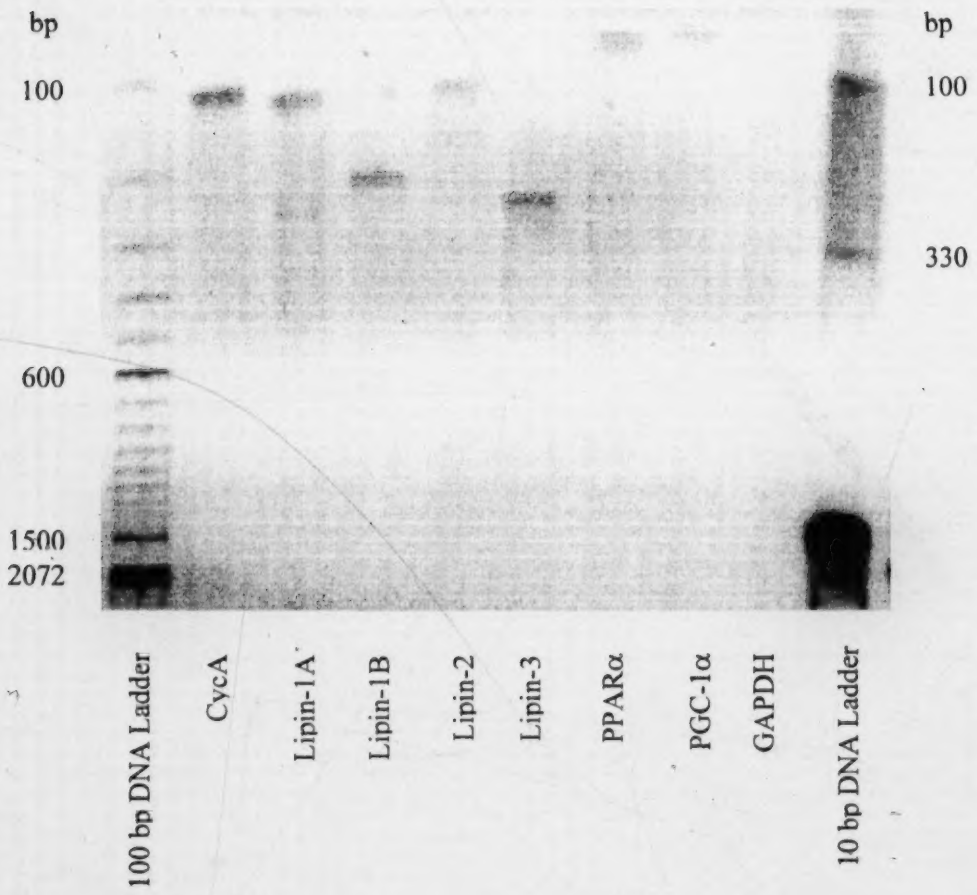

Figure 2.3. The PCR product of different mouse genes of interest amplified by oligonucleotide primers for mouse. The products were separated in 2% agarose gel.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Introduction

In order to investigate the regulation of the expression of different lipins in the liver, primary cultures of mouse and rat hepatocytes were used in the experiments. On one hand, the mouse hepatocytes were used so that what is learned from this work can be related to genetic mouse models that already exist [54, 83], or will be later created. On the other hand, the rat hepatocytes were chosen since earlier work performed on rat hepatocytes had shown significant changes in PAP1 activity when the cells were treated with glucagon, dexamethasone, CPTcAMP and insulin [1, 46].

Primary cultures of mouse or rat hepatocytes were incubated for various times with different combinations of hormones. The cell lysates obtained at the end of treatment were used in real-time RT-PCR measurement, Lipin-1 Western Blot analysis and PAP1 activity assay to determine the lipins mRNA expression, lipin-1 protein expression and the total PAP1 activity, respectively.

3.2 Lipins mRNA Expression

3.2.1 Hormonal regulation of lipin-1 mRNA expression

In mouse hepatocytes, the relative mRNA levels for lipin-1A and lipin-1B were increased by dex and reached a peak after 3 and 4 h respectively (Figs. 3.1A & 3.1C). This dex effect on lipin-1A and lipin-1B was amplified by about 2-fold at their peak by CPTcAMP (Figs. 3.1A & 3.1C), or in the case of lipin-1B by about 1.5 fold by glucagon (Fig. 3.2A). The amplification of the dex effect for lipin-1A by glucagon did not reach statistical

significance. CPTcAMP or glucagon had no significant effect in increasing the mRNA for lipin-1A or lipin-1B compared to the control (DMSO-treated) incubations (Figs. 3.1A, 3.1C & 3.2A). Insulin alone had no significant effect on the relative mRNA concentrations for lipin-1A or lipin-1B compared to the non-treated control. However, it attenuated the effects of dex, and dex with CPTcAMP or glucagon by approximately 50% (Figs. 3.1B, 3.1D & 3.2A).

For rat hepatocytes work, primers for total lipin-1, instead of lipin-1A, and lipin-1B were used since the sequence for these lipins were predicted nucleotide sequences and the primers designed based on the predicted nucleotide sequences did not work properly. In rat hepatocytes, the relative mRNA levels for lipin-1 and lipin-1B in the non-treated controls declined approximately 70% as the incubation proceeded (Figs. 3.3A & 3.3C). Dex increased the level of both lipin-1 and lipin-1B mRNAs relative to the value at zero time of incubation and even more so when compared to the non-treated control at the equivalent time. Their relative mRNA levels reached a peak by 8 h of incubation. CPTcAMP, or glucagon alone had no significant effect on the levels of lipin-1 and lipin-1B mRNA (Figs 3.2B, 3.3A & 3.3C).

As shown in figures 3.2B, 3.3A and 3.3C, CPTcAMP or glucagon appeared to have a slight effect in increasing the dex-induced increase in the levels of lipin-1 and lipin-1B mRNAs. However, the increase of lipin-1 and -1B mRNAs reached statistical significance only at 8 h and 4 h points, respectively. Insulin alone had no significant

effect on lipin-1 or lipin-1B mRNA level. However, it acted antagonistically, significantly decreasing the effects of dex alone, or dex with CPTcAMP or glucagon (Figs. 3.2B, 3.3B & 3.3D).

3.2.1.1 The effect of Actinomycin D and Cycloheximide

Actinomycin D blocked the dex effect on increasing the levels of mRNA for lipin-1A or lipin-1B in mouse hepatocytes (Figs. 3.4A & 3.4B) and for lipin-1 and lipin-1B in rat hepatocytes (Figs. 3.5A & 3.5B). These results demonstrate that transcription is required for increased mRNA expression. In mouse hepatocytes, cycloheximide, which is an inhibitor of protein synthesis, reduced the increase of relative lipin-1A and lipin-1B induced by dex (Figs, 3.4D & 3.4E). On the contrary, the dex-induced expression of mRNAs for lipin-1 and lipin-1B in rat hepatocytes was not decreased by the presence of cycloheximide (Figs. 3.5C & 3.5D). Interestingly, there was an increase in the level of lipin-1 and -1B mRNA concentration when actinomycin D and cycloheximide were used to treat the control dishes. This could possibly result from superinduction, which in some cases could be attributed to decreased mRNA degradation and blockade of translation [84].

3.2.1.2 Relationship of PPARα and PGC-1α mRNA expression to that of lipin-1

In mouse hepatocytes, full induction of hepatic lipin-1 expression under conditions such as fasting requires the presence of PGC-1α [60]. In addition, lipin-1 also interacts physically with both PGC-1α and PPARα [60]. To understand if the effects of dex,

CPTcAMP and insulin on the lipin-1A or lipin-1B mRNA expression depend upon preceding changes in the transcription for PGC-1α and PPARα, the time course and hormonal requirements for PGC-1α and PPARα expression were determined.

In mouse hepatocytes, the increase of relative PGC-1α mRNA level reached maximum after incubating with CPTcAMP for 4 h. Dex alone had no significant effect on the expression of PGC-1α mRNA. However, it synergized the action of CPTcAMP and increased the level of PGC-1α mRNA level by approximately 2-fold (Fig. 3.6A). Insulin did not significantly alter these effects of CPTcAMP and dex + CPTcAMP on PGC-1α mRNA level (Fig. 3.6B).

Dex increased PPARα mRNA expression after 4 h of incubation (Fig. 3.6C). CPTcAMP alone had no effect on PPARα mRNA expression, however it delayed the maximum expression of PPARα to 8 h. Insulin alone had no effect on PPARα mRNA expression and did not significantly alter the effect of dex, or dex + CPTcAMP (Fig. 3.6D).

In rat hepatocytes, CPTcAMP significantly increased PGC-1α mRNA expression, which increased to a greater extent when CPTcAMP was used in combination with dex. The maximum increase of PGC-1α mRNA occurred at 8 h of incubation (Fig. 3.7A). Insulin had no significant effect on the expression of PGC-1α mRNA. Although it seemed like insulin slightly brought down the expression of PGC-1α when the rat hepatocytes were

treated with CPTcAMP and dex + CPTcAMP, the decrease did not reach statistical significance (Fig. 3.7B).

Dex alone increased the expression of PPARα mRNA by 8 h of incubation (Fig. 3.7C), but in contrast to mouse hepatocytes, CPTcAMP partly attenuated the dex effect. As in mouse hepatocytes, insulin alone did not alter the expression of PPARα mRNA (Fig. 3.7D). However, insulin greatly attenuated the dex-induced increase in mRNA for PPARα, whose effect was absent in mouse hepatocytes.

The results from both mouse and rat hepatocytes show that the inductions of PGC-1α and PPARα mRNA expression by CPTcAMP and dex, respectively, occur at the same time or slightly delayed, rather than preceding that for lipin-1A and lipin-1B. Dex alone does not increase PGC-1α mRNA expression and therefore the induction of lipin-1 mRNA production by dex cannot rely on an indirect effect through PGC-1α production. In addition, insulin does not block the increase of PGC-1α mRNA in mouse or rat hepatocytes. Therefore, the insulin effect in decreasing the production of lipin-1A and lipin-1B mRNA cannot be mediated through decreasing the transcriptional regulation of *Lpin1* by regulating PGC-1α expression.

3.2.2 Hormonal regulation of lipin-2 mRNA expression

For mouse hepatocytes, no treatment employed increased the expression of lipin-2 mRNA (Figs. 3.1E & 3.1F). As a matter of fact, the expression of lipin-2 mRNA

decreased during the incubation period in the non-treated controls, and in dex- or insulin-treated hepatocytes which were not different from control. The presence of CPTcAMP alone, or in combination with dex, or insulin maintained the mRNA at the starting level for about 8 h. For rat hepatocytes, lipin-2 mRNA level was reduced by all treatments compared to control over the period of incubation under all conditions (Figs. 3.3E & 3.3F).

3.2.3 Hormonal regulation of lipin-3 mRNA expression

Treatment of mouse hepatocytes with dex increased the relative lipin-3 mRNA concentrations by about 4-fold after 8 h (Fig. 3.1G). CPTcAMP, or insulin alone had no significant effect relative to the non-treated control, but both decreased the dex-induced increase in lipin-3 mRNA (Fig. 3.1H).

In rat hepatocytes, none of the hormonal treatments changed lipin-3 mRNA levels relative to the non-treated control (Fig. 3.3G). There appeared to be a gradual increase in lipin-3 mRNA levels during the 18 h of incubation even in the non-treated control (Fig. 3.3H).

3.2.3.1 The effect of Actinomycin D and Cycloheximide

Treating mouse hepatocytes with actinomycin D or cycloheximide resulted in the inhibition of the dex-induced increase in lipin-3 mRNA (Figs. 3.4C, and 3.4F). Both of these inhibitors also attenuated the lipin-3 mRNA expression in the non-treated control

hepatocytes after 8 h (Figs. 3.4C and 3.4F). One explanation for these results is that the transcription of lipin-3 mRNA in mouse hepatocytes is dependent on the continuous synthesis of an unidentified protein. Another explanation is rapid degradation of existing resting levels of the mRNA.

3.3 Lipin-1 protein expression

According to the results obtained from the studies, which indicated that dex induced increases in lipin-1 mRNA levels, we investigated whether this resulted in an increase in the expression of lipin-1 protein.

Dr. Meltem Sariahmetoglu used the cell lysates collected from the mouse and rat hepatocyte experiments to determine the level of lipin-1 protein expression. The quality of the lipin-1 antibody was determined using V5-tagged recombinant lipin-1A, -1B, -2, and -3, and was shown not to cross-react with lipin-2 and lipin-3 (Fig. 8A from the article attached to the appendix). It was also tested against proteins isolated from wild-type and *fld* mice adipose tissues, which served as positive and negative controls respectively (Fig. 8B from the article attached to the appendix). These results showed that lipin-1 antibody can be used to detect lipin-1 in Western blot analysis.

The Western blot analysis for lipin-1 used lysates from mouse and rat hepatocytes incubated with a combination of hormones consisting of CPTcAMP, dex and ins for 8 and 12 h, respectively. The results are shown in Figs. 8B & 8C from the article attached

to the appendix. Quantification of the results revealed that dex increased lipin-1 protein in hepatocytes of both species. This dex-effect is enhanced when dex was used in combination with CPTcAMP. Insulin alone did not have any effect on the expression of lipin-1 protein, but it significantly attenuated the effect of dex+CPTcAMP.

As shown in the Western blot of lipin-1 (Fig. 9A from the article attached to the appendix), actinomycin D and cycloheximide blocked the increases in mouse and rat lipin-1 protein levels induced by dex. This was reflected in the lack of increase in PAP1 activity (Fig. 3.8). These results demonstrated that the dex-induced increase in lipin-1 expression and PAP1 activity depends upon both transcription and translation.

3.4 PAP1 activity

PAP1 activity was measured in order to investigate the relationship with the increases of lipin mRNA level, lipin-1 protein level, and the PAP1 activity. In mouse hepatocytes, there was an increase in PAP1 activity by approximately 1.7 fold, when compared to the activity at time zero, after incubating with dex for 8 h (Fig. 3.9A). This increase in PAP1 activity was further increased to approximately 2.3 fold when the hepatocytes were treated with dex + CPTcAMP (Fig. 3.9A). The increases observed remained relatively constant until at least 16 h. CPTcAMP alone as well as insulin alone did not result in a significant change in PAP1 activity (Figs 3.9A & 3.9B). However, insulin partially attenuated the effect of dex and dex + CPTcAMP (Fig. 3.9B).

In rat hepatocytes, there was an increase in PAP1 activity by approximately 2.5 fold when the cells were incubated for 12 or 18 h, compared to the activity at the beginning of incubation (Fig. 3.9C). CPTcAMP alone did not significantly alter PAP1 activity, but it prevented the decline of up to 70% in activity that occurred at 18 h in the untreated controls or in insulin-treated cells (Fig. 3.9C). These results are compatible with the known stability of PAP1 activity in rat hepatocytes. PAP1 activity in untreated or insulin-treated cells has a half-life of 5-7 h, and this is increased to 12 h by glucagon (through cAMP) [46]. Incubating the hepatocytes with dex + CPTcAMP resulted in a significant increase in PAP1 activity of approximately 4 fold after 12 or 18 h of incubation (Fig. 3.9C). Insulin acted antagonistically with dex and dex + CPTcAMP (Fig. 3.9D) and decreased the relative PAP1 activity.

The increase of lipin-1 mRNA precedes the increase of lipin-1 protein and PAP1 activity as expected. The peak of lipin-1A and -1B mRNA in mouse hepatocytes and lipin-1 and -1B in rat hepatocytes occurred at 4 and 8 h, respectively, after incubation whereas the increase in PAP1 activity peaked at 8 and 12 h. As seen in Fig. 3.1 and 3.3, there was a significant drop in the level of lipin-1A and -1B mRNA in mouse hepatocytes and lipin-1 and -1B mRNA in rat hepatocytes 4 h after the level of lipin-1 mRNA has reached its peak. However, the level of PAP1 activity of both mouse and rat hepatocytes as shown in figure 3.9 were sustained. This drop in the mRNA of different lipin-1 isoforms after the dex induction suggested that there was a greater turnover for lipin-1 mRNA than that for lipin-1 protein and PAP1 activity.

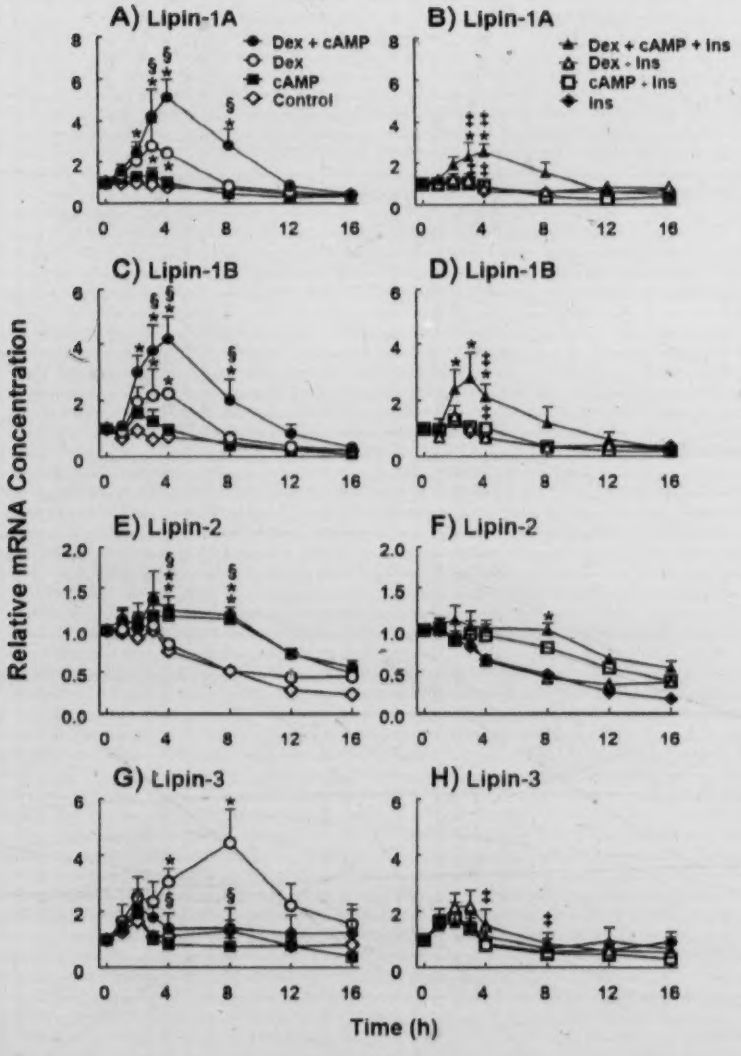

Figure. 3.1. Interaction of dexamethasone, cAMP and insulin in controlling mRNA expression for lipin in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dexamethasone (*dex*), 100 μM CPTcAMP (*cAMP*), 100 nM insulin (*ins*) alone or in combination as indicated. Relative mRNA concentrations for the different lipins were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± S.E.M. for 3-15 independent experiments. The significance of the differences ($p<0.05$) are indicated as follows: *, different from the untreated control value; §, dex alone different from dex + CPTcAMP treatment; and ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

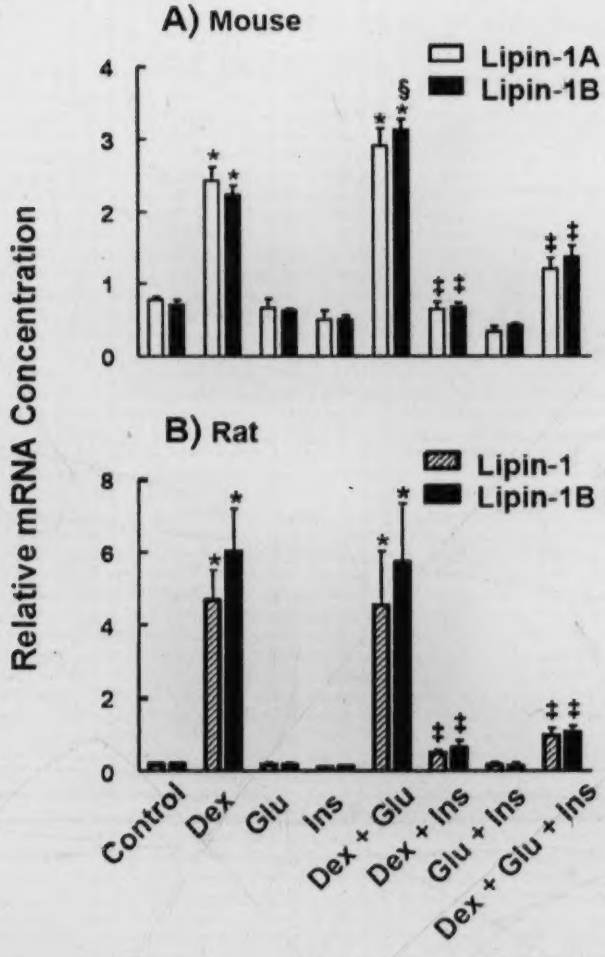

Figure. 3.2. Interaction of dexamethasone, glucagon and insulin in controlling mRNA expression for lipin-1 in mouse and rat hepatocytes. Mouse (*A*) and rat (*B*) hepatocytes were incubated for 4 and 8 h, respectively, with 100 nM dexamethasone (*dex*), 10 nM glucagon (*glu*), 100 nM insulin (*ins*) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are means ± S.E.M. for 3 to 15 independent experiments for the mouse and 3 to 8 experiments for the rat. The significance of the differences ($p<0.05$) are indicated as follows: *, different from the untreated control value; §, dex alone value is significantly different from dex with CPTcAMP treatment; ‡, incubation with insulin decreases the effect of dex alone or dex + glucagon.

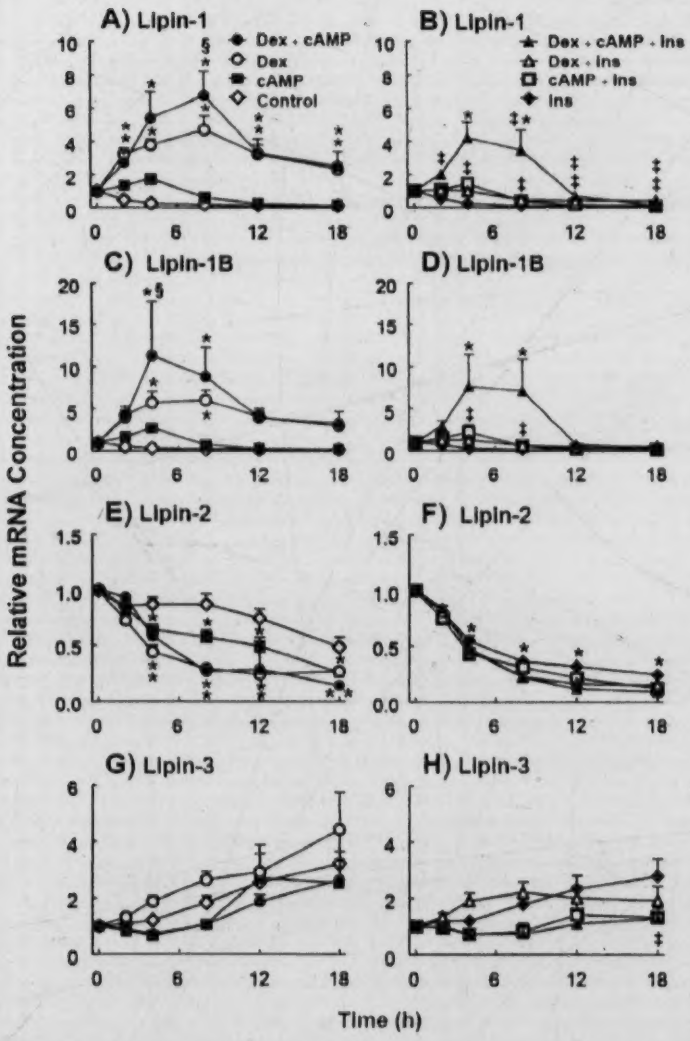

Figure. 3.3. Interaction of dexamethasone, cAMP and insulin in controlling mRNA expression for lipin in rat hepatocytes. Rat hepatocytes were incubated for various times with 100 nM dexamethasone (*dex*), 100 μM CPTcAMP (*cAMP*), 100 nM insulin (*ins*) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± S.E.M. for 3 to 8 independent experiments. The significance of the difference ($p<0.05$) are indicated as follows: *, different from the untreated control value; §, dex alone different from dex with CPTcAMP treatment; and ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

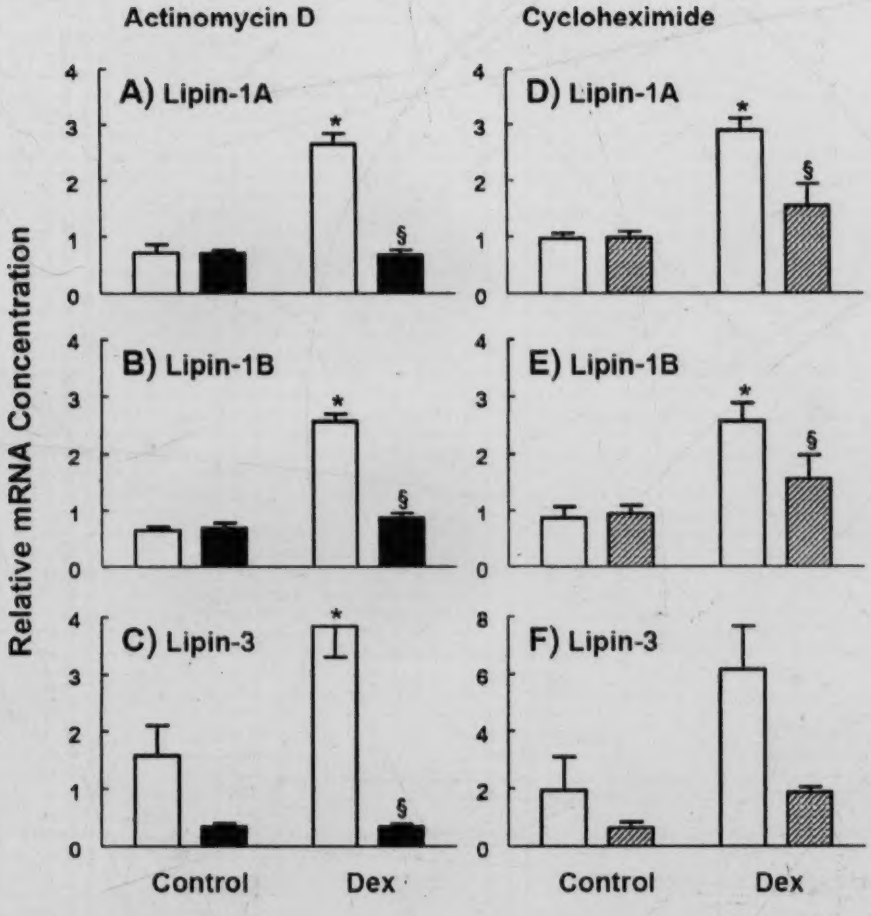

Figure. 3.4. Effects of actinomycin D and cycloheximide on the dexamethasone-induced increase in mRNA for lipin-1A, -1B and -3. Mouse hepatocytes were treated with or without dexamethasone (*dex*) in the presence, or absence of 10 μg/ml actinomycin D (cells were also pre-incubated for 30 min with actinomycin D), or 5 μg/ml cyclohexamide. For measuring mRNA production for lipin-1A and -1B, or lipin-3, the hepatocytes were incubated for 4h, or 8h, respectively, as decided from Fig. 1. White columns show values for incubations in the absence of inhibitor, whereas black and hatched columns indicate the presence of actinomycin D or cycloheximide, respectively. Results are means ± S.E.M. for three independent experiments, except for Panel F where means ± ranges are shown for two experiments. The significance of the differences ($p<0.05$) were evaluated with a Student's t test and are indicated as follows: *, dex treatment different from the untreated control value; §, the actinomycin D, or cycloheximide result is different from the equivalent incubation without these inhibitors.

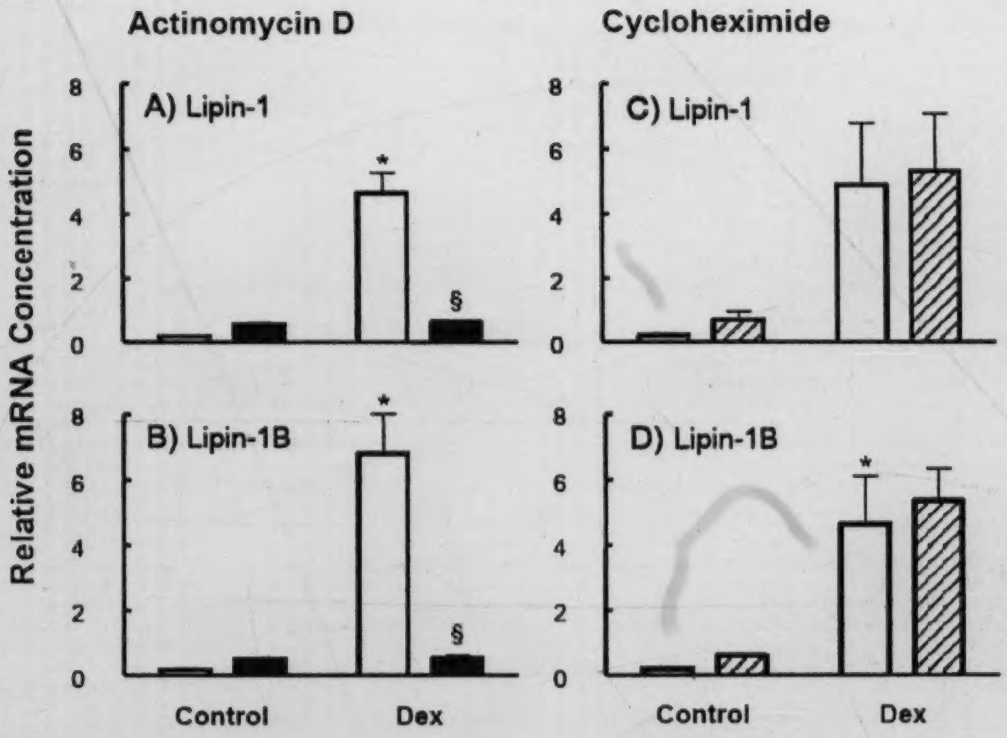

Figure. 3.5. Effects of actinomycin D and cycloheximide on the dexamethasone-induced increase in mRNA for lipin-1, and -1B. Rat hepatocytes were treated with or without dexamethasone (*dex*) in the presence, or absence of 10 μg/ml actinomycin D (cells were also pre-incubated for 30 min with actinomycin D), or 5 μg/ml cyclohexamide. For measuring mRNA production for lipin-1, lipin-1B, and lipin-3, the hepatocytes were incubated for 8h. White columns show values for incubations in the absence of inhibitor, whereas black and hatched columns indicate the presence of actinomycin D or cycloheximide, respectively. Results are means ± S.E.M. for three independent experiments. The significance of the differences ($p<0.05$) were evaluated with a Student's t test and are indicated as follows: *, dex treatment different from the untreated control value; §, the actinomycin D, or cycloheximide result is different from the equivalent incubation without these inhibitors.

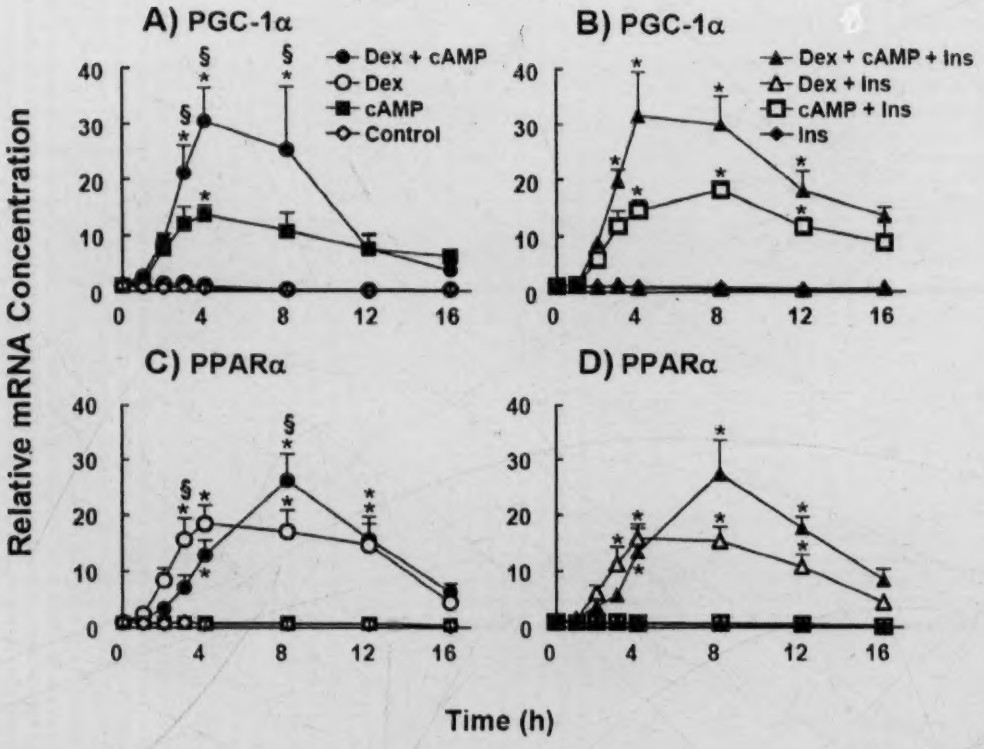

Figure. 3.6. Interaction of dexamethasone, cAMP and insulin in controlling mRNA expression for PPARα and PGC-1α in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dexamethasone (*dex*), 100 μM CPTcAMP (*cAMP*), 100 nM insulin (*ins*) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (*A* and *B*) and PPARα (*C* and *D*) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± S.E.M. for 3 independent experiments. The significance of the differences ($p<0.05$) are indicated as follows: *, different from the untreated control value; and §, dex alone different from dex + CPTcAMP treatment.

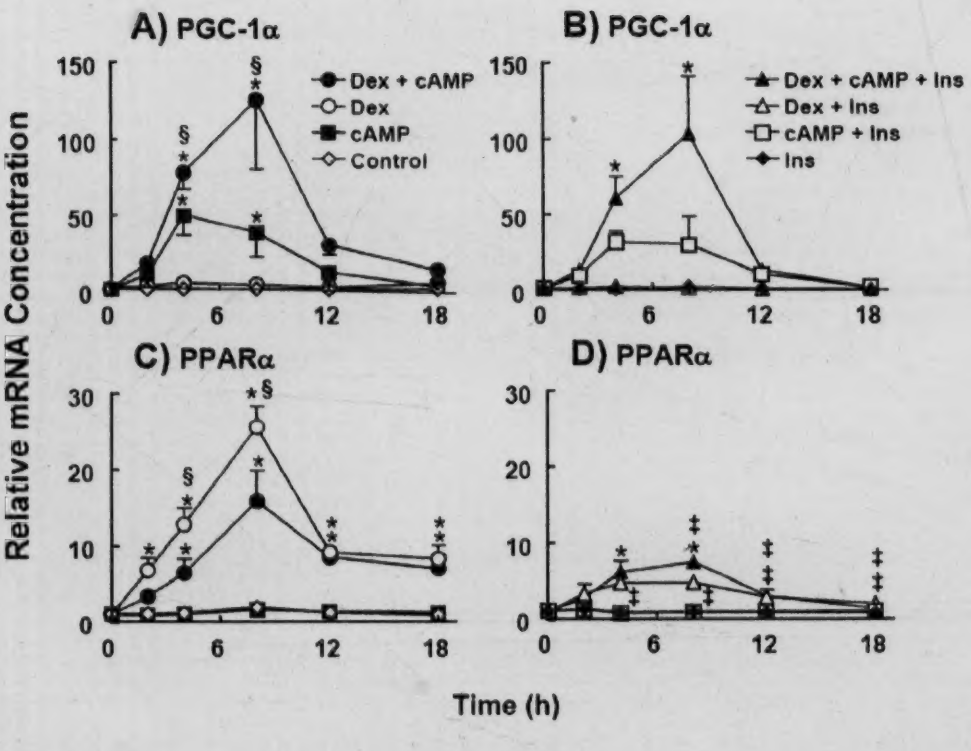

Figure. 3.7. Interaction of dexamethasone, cAMP and insulin in controlling mRNA expression for PPARα and PGC-1α in rat hepatocytes. Rat hepatocytes were incubated for the times shown with 100 nM dexamethasone (*dex*), 100 μM CPTcAMP (*cAMP*), 100 nM insulin (*ins*) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (*A* and *B*) and PPARα (*C* and *D*) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± S.E.M. for 3 independent experiments. The significance of the differences ($p<0.05$) are indicated as follows: *, different from the untreated control value; §, dex alone different from dex + CPTcAMP treatment; and ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

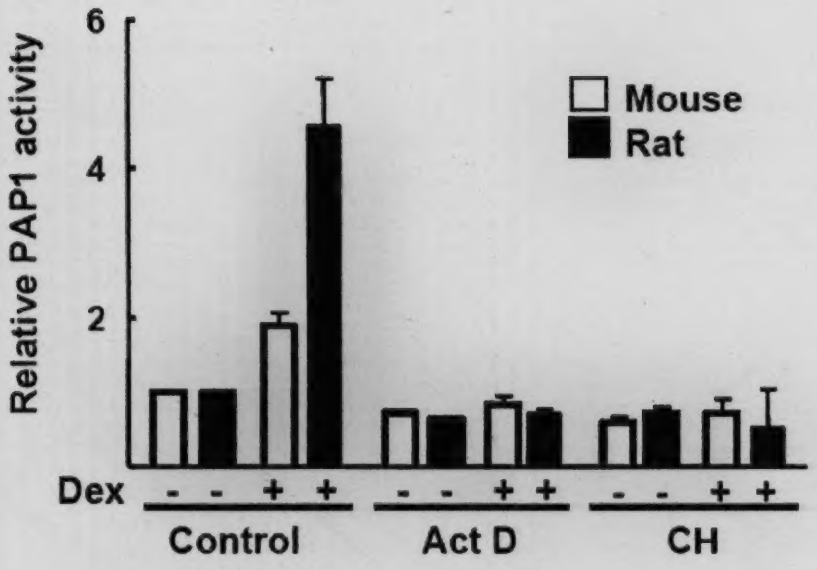

Figure. 3.8. Actinomycin D and cycloheximide block the dex-induced PAP1 activity. Mouse and rat hepatocytes were pre-incubated with or without 10 μg/ml actinomycin D (*Act D*) or 5 μg/ml cycloheximide (*Cyclo*) for 30 min. The inhibitors were maintained in the subsequent incubations in the presence or absence of 100 nM dex, which for mouse and rat hepatocytes were 8 h and 12 h, respectively. These times were based upon the results in Figs. 3.1 and 3.3. The figure shows PAP1 activity relative to the equivalent incubation in the absence of inhibitors or dex. Results are means ± S.E.M. for 3 independent experiments with mouse hepatocytes and means ± ranges for 2 experiments with rat hepatocytes. The measurement of PAP1 activity was partly performed by Dr. Meltem Sariahmetoglu and Mr. Jay Dewald.

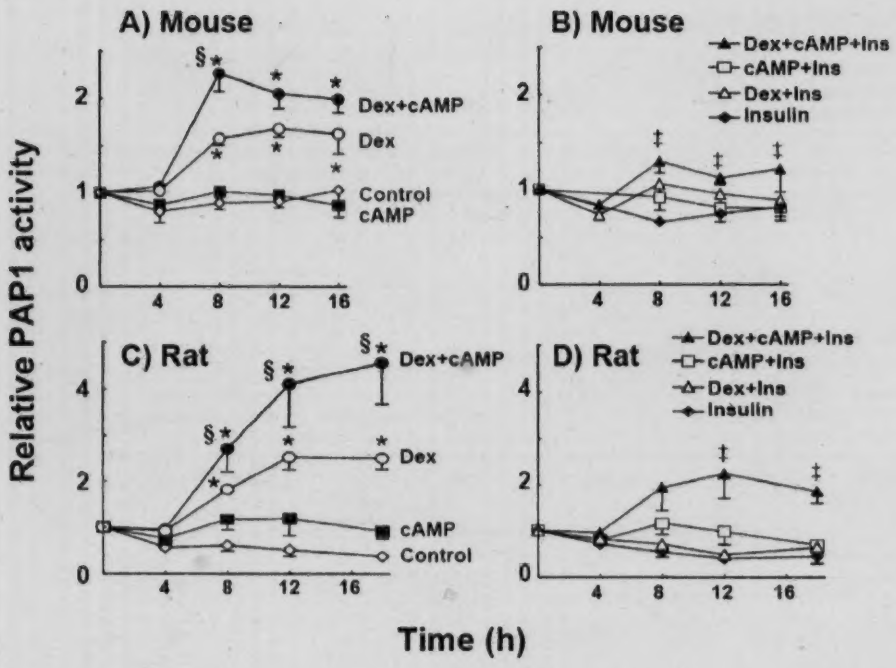

Figure. 3.9. Interaction of dexamethasone, glucagon and insulin in controlling PAP1 activity in mouse and rat hepatocytes. Mouse (*A* and *B*) and rat (*C* and *D*) hepatocytes were incubated for various times with 100 nM dexamethasone (*dex*), 100 μM CPTcAMP (*cAMP*), 100 nM insulin (*ins*) alone or in combination as indicated. The average PAP1 specific activity at the beginning of the incubation was 32 ± 15 (n = 7) and 36 ± 12 (n = 5) nmol DAG produced/min per mg protein for mouse and rat hepatocytes, respectively. The results are expressed relative to the initial untreated value, which was normalized to 1. Results are means ± S.E.M. for 3-9 independent experiments. The significance of the differences ($p<0.05$) are indicated as follows: *, different from the untreated control value; §, dex alone value is significantly different from dex + CPTcAMP treatment; ‡, incubation with insulin decreases the effect of dex alone or dex + CPTcAMP. The measurement of lipin-1 expression and PAP1 activity were partly performed by Dr. Meltem Sariahmetoglu and Mr. Jay Dewald.

3.5 Discussion

The identification of lipin proteins as PAP1 enzymes [30] makes it possible to investigate the modulation of PAP1 activity that occurs in response to various physiological and pathological conditions at the molecular level. Donkor et al. have demonstrated that the liver expresses lipin-1A, -1B, -2, and -3 [10]. This enables us to continue the study on how each lipin responds to glucocorticoid, glucagon, and insulin. Moreover, it was very interesting to understand the functions of individual lipins and the reason for changes in the composite PAP1 activity that had been described previously [1, 37, 46].

The result from real-time RT-PCR experiments performed using mouse or rat hepatocytes shows that the hormonal control is similar between the two rodent species. The two splice variants of *Lpin1* gene, lipin-1A and lipin-1B, are induced at the transcriptional level by dexamethasone, a synthetic glucocorticoid. This dex action, especially in mouse hepatocytes, was synergized by glucagon through cAMP. Insulin antagonized these effects of glucagon and dexamethasone in both rat and mouse hepatocytes. Insulin can block the effects of glucagon by stimulating phosphodiesterase activity and diminishing the increase in cAMP. In these experiments, CPTcAMP, which is a synthetic analog of cAMP that cannot be readily degraded by phosphodiesterase, was used [85]. The result shows that insulin exerts similar antagonistic effects on *Lpin1* transcription that were produced by CPTcAMP. This indicates that insulin exhibits a more direct effect on lipin-1 expression than by just decreasing cAMP concentrations.

Despite their difference in terms of localization, there is no evidence that the combination of hormones used regulates the relative expression of the two lipin-1 splice isoforms in hepatocytes. The dex-induced increase in PAP1 activity depended upon an increase in the expression of *Lpin1* gene and lipin-1 mRNA. This increase is subsequently followed by an increase in lipin-1 synthesis and protein expression, which was directly shown by the Western Blot analysis presented in the article attached. Additional evidence is provided to support this conclusion in the experiments where actinomycin D and cycloheximide were used to block the increase of lipin-1 mRNA and lipin-1 protein expressions, respectively.

The results obtained from the real-time RT-PCR experiments revealed that lipin-2 and lipin-3 expression is differently regulated compared to lipin-1. Incubation of mouse hepatocytes with dex resulted in a time-dependent increase in lipin-2 mRNA when compared to control. In rat hepatocytes, on the contrary, a decrease in lipin-2 mRNA was observed. A significant increase in lipin-3 mRNA was observed in response to dex in mouse hepatocytes, and this was attenuated by CPTcAMP. In rat hepatocytes, on the other hand, there was no significant change in lipin-3 mRNA level when treated with dex or dex + CPTcAMP in comparison to control. These results demonstrate that the increase of lipin-2 and lipin-3 transcription did not contribute to the increased PAP1 activity, which depended upon increased transcription and translation, induced by dex + CPTcAMP. Despite the fact that there was an increase in lipin-3 mRNA induced by dex

observed in mouse hepatocytes, the induction of lipin-3 mRNA parallels with the increase in PAP1 activity at 8 hours after incubation. Therefore it is evident that lipin-3 is not contributing to PAP1 activity at the same time as lipin-1, but it is possible that lipin-3 contributes to PAP1 activity at the later time. At the moment, the measurement of the level of lipin-2 and lipin-3 protein is not possible due to the lack of suitable antibodies needed to detect the proteins in crude liver lysates.

The conclusion that an increase in PAP1 activity induced by dex and dex + CPTCAMP is accounted for by an increase in lipin-1 expression is strongly supported by studies *in vivo*. There was a lack of an increase in PAP1 activity of the livers of fasted *fld* mice, which do not express lipin-1, compared to approximately 2-fold increases in control mice [52]. Lipin-1 transcription and lipin-1 protein levels in the livers of wild-type mice are increased after fasting or dex injection [60]. In an experiment done by our colleagues, there was an increase in the level of expression of lipin-1A, -1B, and PGC-1α mRNA, but not lipin-3 mRNA in fasted mice [86]. To our surprise, the level of lipin-2 mRNA was doubled in the fasted mice livers [86]. This is unlikely to have occurred by GC action, based upon my hepatocyte work. It could have resulted from a cAMP effect, since this second messenger seemed to increase the concentration of lipin-2 mRNA in mouse hepatocytes relative to the non-treated or dex-treated cells. However, the physiological significance of the increased lipin-2 mRNA in fasted mice is uncertain, due to the lack of increase in hepatic PAP1 activity in *fld* mice after fasting [52]. This evidence supports the conclusion that increased lipin-1 mRNA and protein expression account for the increase

in PAP1 activity, which occurs when there is increased GC level relative to the insulin level. This is supported by the discovery by Zhang et al. that the lipin-1 promoter contains a functional GC response element and that the dex effect is mediated through the GC receptor [87]. These authors recently characterized the position of glucocorticoid response element (GRE) in the *Lpin1* promoter using reporter gene transcription, electrophoretic mobility shift, and chromatin immunoprecipitation assay. They showed that the GC receptor binds to the *Lpin1* GRE and this leads to a transcriptional activation in adipocytes and hepatocytes. This molecular mechanism of lipin-1 induction suggests that lipin-1 may serve as an important mediator of glucocorticoid effect on lipid and lipoprotein metabolism. The work published by Zhang et al. [87] has revealed that PAP1 activity in adipocytes can be induced by glucocorticoids through a GRE. While what is learned from the article contradicts the basis of the original hypothesis, it was not until half a year after my work was completed when the article by Zhang et al. became available.

In addition, there could be other factors that also contribute to the increase in PAP1 activity observed. Glucagon, through cAMP, increases the half-life of GC-induced PAP1 activity by about 1.7 fold, from 7 to 12 h [46]. This could contribute to the increased lipin-1 protein expression produced by dex + CPTcAMP. Second, CPTcAMP could affect PAP1 activity through its phosphorylation [22, 37]. Brindley et al. have provided indirect evidence for this prior to the discovery of the lipins. Direct evidence regarding phosphorylation of yeast PAP1 (also known as SMP2 and PAH1) was recently described

[88]. According to work done in adipose tissues [51, 52], the level of lipin-1 phosphorylation is controlled by the balance of signaling from insulin versus cAMP. The changes in phosphorylation of lipin-1 were not reflected in changes in PAP1 activity as measured in vitro [52]. However, there was evidence showing that treatment of liver cytosol with phosphatases did decrease total PAP1 activity [22]. The major effect of lipin-1 phosphorylation appears to be to regulate its subcellular distribution [52] and thereby regulates its physiological expression and subsequently activity [31].

Thirdly, it could be that lipin-1 and PAP1 activity is regulated through interaction with other proteins, such as PGC-1α and PPARα [60]. Lipin-1 has also been shown to serve nuclear functions by acting as an inducible coactivator of the PGC-1α-PPARα circuit to increase hepatic fatty acid oxidation and ketogenesis [60]. Not only does lipin-1 amplify signaling by PGC-1α and PPARα, but PGC-1α is required as a coamplifier of lipin-1 expression in the liver. Therefore, induction of lipin-1 expression in fasted or in dex-treated mice is partially attenuated when PGC-1α is totally deficient, indicating a partial dependence on PGC-1α. Hepatic lipin-1 expression is also increased in type 1 and type 2 diabetes, conditions in which PGC-1α expression is elevated [60]. The result which shows that cycloheximide partially inhibits the dex-induced production of lipin1 mRNA in mouse hepatocytes indicated that the induction of cotranscriptional regulators such as PGC-1α, may be required for full lipin-1 expression. A similar effect of cycloheximide was not observed in rat hepatocytes. This might suggest that the level of endogenous co-transcriptional regulators in the rat hepatocytes is sufficiently high for sustaining the level

of lipin-1 transcriptional induction. Significantly, CPTcAMP had only a marginal effect in increasing the dex-induced production of lipin-1 mRNA in rat hepatocytes, whereas the effect was more marked in mouse hepatocytes.

As reported previously [89, 90], PGC-1α mRNA was increased when mouse or rat hepatocytes were treated with CPTcAMP. Dex alone had no significant effect on the level of PGC-1α mRNA, but it greatly synergized CPTcAMP action. On the contrary, dex effectively increased PPARα mRNA expression as earlier described [91], and there was little if any effect of CPTcAMP. These changes in the level of PGC-1α and PPARα are compatible with what was observed in starvation and diabetes [89, 90]. Although insulin decreases the signaling effects of PGC-1α in vivo [89, 90], there was no significant effect of insulin in directly decreasing the expression of PGC-1α mRNA in mouse or rat hepatocytes. The results obtained for PGC-1α agree with previous work with mouse hepatocytes [90]. Although lipin-1 expression depends partly on the presence of PGC-1α [60], the dex + CPTcAMP effect in increasing the mRNA for PGC-1α and PPARα mRNA do not precede the maximum expression of lipin-1. In addition, the inhibition of lipin-1 mRNA expression by insulin probably results from an action on transcription of the *Lpin1* gene, because insulin did not block the stimulation of PGC-1α. In the case of PPARα, there was an inhibition of the dex-induced effect in rat hepatocytes, but not in mouse hepatocytes.

In liver, the interaction of lipin-1 with PGC-1α and PPARα promotes the transcriptional regulation of enzymes involved in β-oxidation, and this is also a response to starvation or diabetes [60]. Brindley et al. proposed that the GC-induced increase in PAP1 activity could be an adaptive response to protect the liver against an increased FA load on lipotoxicity in starvation and diabetes [31, 37]. However, TAG accumulation in the liver could itself also be lipotoxic [92]. The work done here has provided evidence that lipin-1 is responsible for the GC-induced increase in PAP1 activity, which was previously shown to increase hepatic glycerolipid synthesis [36]. As the concentration of FA increases and exceeds β-oxidation capacity, PAP1 translocates to ER membranes to facilitate the storage of excess FA as TAG. The accumulated fat droplets results in steatosis [31]. The FA-induced translocation of lipin-1 also occurs in adipocytes and this facilitates limited FA recycling to TAG during active lipolysis [52]. TAG synthesis in the liver should not be regarded as a process antagonizing β-oxidation. It should be viewed as a companion pathway since the stored TAG is turned over and FA used for oxidation [31]. Besides facilitating TAG synthesis, increased lipin-1 expression also promotes the transcription of key enzymes in FA oxidation [60].

Another possibility is that the stored TAG is hydrolyzed, and together with exogenous FA, they are re-esterified for VLDL production. The secretions of TAG and apolipoprotein B are also increased by GC, and insulin antagonizes this action [93-95]. Hepatic PAP1 activity is also positively correlated with circulating TAG [31, 40, 95], and

the changes in the level of lipin-1 are probably partly responsible for the regulation of VLDL secretion.

Recent work done by Bou Khalil et al. (submitted to JLR) [96] have shown that the effects of GC in increasing hepatic expression of lipin-1A and lipin-1B are involved in the stimulation of hepatic TAG synthesis and increased secretion of TAG and apolipoprotein B. They provide evidence that after the expression of lipin-1A and lipin-1B in the rat liver hepatoma, McA-RH7777 cells, PAP1 activity increased as did the synthesis and secretion of DAG, TAG and PC when the cells were in basal condition or supplemented with oleate. They also reported that in the latter condition, the secreted TAG was mainly associated with the larger and less dense $VLDL_1$ and smaller and denser $VLDL_2$. In addition, they showed that there was an increase in the secretion efficiency and a decrease in the intracellular degradation of apoB100 when lipin-1A and lipin-1B were expressed. These results are consistent with the levels of lipin-1 controlling the assembly and secretion of VLDL. In a report where DGAT was overexpressed in mouse liver, there was an increase in TAG content of the liver but no increase in the production rate of VLDL TAG or apoB [97]. This finding contradicts the common belief that hepatic TAG synthesis modulates the VLDL production. Because VLDL production and secretion requires other lipids and proteins such as PC, PE, and apoB100, an increase of DGAT activity and TAG alone need not influence the production of VLDL. Unlike DGAT whose activity is at the ultimate step in TAG synthesis, the activity of lipin is at the branch point of both TAG synthesis and phospholipid synthesis. Therefore lipin-1

appears to be an important regulator of both TAG and phospholipid biosyntheses since it controls the synthesis of DAG substrate for TAG, PC and PE production and can decrease the degradation of apoB100.

It is also possible that the responses of hepatic PAP1 to GC, glucagon, and insulin are coregulated with those of enzymes involved in controlling gluconeogenesis [1, 31]. Transcription of key gluconeogenic enzymes is also regulated through PGC-1α and PPARα. The coregulation of lipin-1 expression and the physical interactions of lipin-1 with PGC-1α and PPARα [60] might participate in modulating and integrating gluconeogenesis with the increased capacity for hepatic TAG synthesis and β-oxidation in starvation and diabetes. This mechanism may account for the 40% decrease in hepatic glucose production during fasting in liver of *fld* mice [62].

Overexpression of lipin-1 in the liver can be beneficial for patients who suffer from diabetes. Because diabetes patients develop insulin resistance and have an increased circulating fatty acid load to the liver, the increased lipin-1 expression and hence PAP1 activity can increase the liver's capacity for converting the toxic fatty acids into TAG. This could be protective. On the other hand, it is important to take into account the fact that diabetic patients develop hyperinsulinemia because of insulin resistance and insulin normally attenuates lipin-1's PAP1 activity. In this sense, the increase in hepatic PAP1 (lipin-1) activity is a marker for dysfunctional metabolism that predispose to cardiovascular diseases [9].

Due to the fact that enhanced lipin-1 expression in the adipose tissue can decrease insulin resistance, it could be beneficial for type-2 diabetes patients who developed insulin insensitivity to have an overexpression of lipin-1 in their adipocytes. This is often achieved by administration of PPARγ agonist, Rosiglitazone [98]. However, increased lipin-1 expression in adipose tissue can lead to increased fat mass. Therefore type-2 diabetes patients ought to beware of this side effect. Similar to enhanced lipin-1 expression in adipocytes, overexpression of lipin-1 in skeletal muscle can also lead to obesity. However, unlike in the adipose tissue, overexpression of lipin-1 in skeletal muscle can result in insulin resistance.

To conclude, *Lpin1* transcription in mouse and rat hepatocytes is induced by GC. cAMP synergizes this effect and insulin antagonizes it. There was no significant increase in the expressions of lipin-2 in both mouse and rat hepatocytes. Even though there was an increase in lipin-3 mRNA in mouse hepatocyte at a single time point, a similar increase was absent in rat hepatocytes. Thus it is the dex-induced increase in lipin-1 expression, but not lipin-2 and lipin-3, which contributes to the increased PAP1 activity in the liver. The increase in lipin-1 mRNA of both splice variants, lipin-1α and lipin-1β, subsequently results in an increased lipin-1 protein expression as well as PAP1 activity. This increase in PAP1 activity was also observed in starvation and diabetes. The FA-stimulated association of lipin-1 with the nucleus probably allows it to regulate transcription and increase FA oxidation in starvation and diabetes. Increased lipin-1 expression also

increases the liver capacity for glycerolipid synthesis and VLDL secretion. This is achieved as the accumulated FA causes lipin-1 to translocate to the ER, where TAG is produced. In turn, TAG builds up causing steatosis observed in starvation, diabetes, ischemia, toxic conditions, and ethanol intoxication.

CHAPTER 4

FUTURE DIRECTIONS

4.1. Mechanism of lipin transcriptional regulation

One area of study that follows my work would be to identify how cAMP and insulin regulate the induction of lipin-1 expression by glucocorticoids. Dex promotes the transcription of *Lpin1* through an identified glucocorticoid response element [87]. Based on computer prediction analysis, the putative cAMP response element binding-protein (CREB) and forkhead box A2 (FOXA2) sites were found in the lipin-1 nucleotide sequence (as shown in Fig 4.1). The CREB site is a well-characterized site where a transcription factor called cAMP response element binding protein (CREB) binds to the specific DNA sequence and alters the transcription level of specific genes. CREB is a member of a large family of transcription factors with a basic region leucine zipper (bZIP), which mediates both sequence-specific binding and dimerization. CREB is activated through phosphorylation on a serine residue in response to protein kinase A, which is activated by cAMP. The phosphorylated CREB then binds to CREB-binding protein (CBP), a co-activator which then makes contact with other components resulting in the induction of transcription [99, 100].

FOXA2 is a hepatocyte nuclear factor, which is a subset of transcription factors. We have learned that insulin attenuates the GC and cAMP stimulation of transcription for lipin-1 in mouse and rat hepatocytes, and from previous work that the regulation of PAP1 expression by insulin, GC and cAMP parallels that of key enzymes of gluconeogenesis and the increased capacity for β–oxidation [1]. Insulin suppresses the expression of several key enzymes involved in gluconeogenesis through the forkhead transcription

factor, FOXO1 [89, 90]. The insulin signaling pathway also regulates Foxa2. In the case of β-oxidation and ketogenesis, insulin regulates the activity of Foxa2 through Akt-mediated phosphorylation, which promotes its nuclear exclusion and prevents the stimulation of transcription [101]. Because lipin-1 expression is linked to increased FA supply and β-oxidation, Foxa2/Hnf3 sites [102] were searched for in the lipin-1 promoter and the putative sites were found (Fig. 4.1).

Future work will involve the expression of various constructs of lipin-1 promoter segments linked to the luciferase reporter (gift from Dr. Karen Reue) into RH7777 rat hepatoma cells. The constructs which were generated at different lengths and were expressed relative to the lipin-1 transcription start site are as follow: -2.045 kb, -1.6 kb, -0.95 kb, -0.8 kb, -0.65 kb, and -0.25 kb. These constructs have been used in collaborative work with Dr. Reue to identify the GRE sites required for the regulation of lipin-1 transcription [87]. The GRE site is found to be located within approximately the first 300 bp of the gene promoter. Since this GRE site is near the transcription start site, it will be possible to study the effects of other transcription factor binding sites which are situated on the promoter upstream of the GRE site, while in the same time being able to induce lipin-1 expression by dex. Due to the fact that RH7777 rat hepatoma cells are easier than hepatocytes to transfect, the experiment will be carried out in these hepatoma cells. However, electroporation will be used to confirm the result in hepatocytes.

After transfection, the RH7777 cells would then be treated with dex and compounds of interest such as CPTcAMP or insulin and the level of luciferase activity can be measured. It is predicted that CPTcAMP will increase the dex-induced expression of luciferase activity while insulin will attenuates it. The results obtained from this experiment will give information on the mechanism for the regulation of lipin-1 transcription by cAMP and insulin. Once the putative C/REB and Foxa2 sites have been determined, the next step would be an identification of these response elements. Mutation study will determine if the putative C/REB and Foxa2 sites identified earlier are required for the regulation of lipin-1 by cAMP and insulin.

2. Lipins mRNA Stability

My work identified how glucocorticoids interact with glucagon and insulin to control the transcription of the *Lpin-1* gene. The other component that would change mRNA for lipin-1A and lipin-1B levels would be the hormonal control of mRNA degradation.

To investigate the stability of lipin mRNA, the primary culture of hepatocytes will be treated with actinomycin D to inhibit the synthesis of new mRNA. Then, they will be treated with compounds of interest such as glucagon, dexamethasone, CPTcAMP and insulin. The relative concentration of lipin mRNA will be measured over in a time course and the half-life for the mRNA will be calculated from each treatment and then compared with the non-treated control to see if any hormonal treatment results in an alteration of the mRNA half-life.

3. Western blotting for lipin-2 and lipin-3

Our laboratory has commissioned the production of antibodies for lipin-2 and -3 that give satisfactory Western blots with recombinant lipins expressed in HEK 293 cells. One of the possible next steps is to investigate directly whether there is a change in the level of lipin-2 and -3 protein expression following the hormonal and agonist treatment. However, my work and that from *fld* mice indicates that lipin-1 is the glucocorticoid-inducible lipin. Therefore, it is unlikely that the results obtained from this experiment will contribute to the increased PAP1 observed when hepatocytes are treated with GC.

4. Lipin protein degradation

Previous studies have shown that there was a decrease in the decay of PAP1 activity when the hepatocytes were treated with 10 nM glucagon or cGMP in the presence of cycloheximide to prevent new proteins including PAP1 synthesis. Conversely PAP1 activity declined more quickly when 500 pM insulin was used [42]. A pulse-chase experiment can now be carried out to study the degradation of the lipins by employing the anti-lipin-1, -2, and -3 antibodies which are now available. In the pulse period, the cells will be treated with [^{35}S]methionine/cysteine followed by replacement with a non-radioactive methionine/cysteine medium. Compounds of interest such as glucagon, dexamethasone, CPTcAMP and insulin will be added to the medium at the beginning of the chase period and the cell lysate will be collected over the chase period. The three lipins will be immunoprecipitated and separated by SDS-PAGE. Scintillation counting of radio-labeled lipin proteins will be used to determine the amount of each ^{35}S-labelled

lipin, allowing determination of the effect that each hormone has on the half-life of the different lipins.

5. ApoB and MTP mRNA expression

Previous studies have shown that glucocorticoids can increase PAP1 activity and hence the capacity of the liver to synthesize TAG [1]. Furthermore, glucocorticoids can also stimulate the production of apoB and drive the secretion of VLDL [93, 94, 103]. By contrast, insulin attenuates the glucocorticoid stimulation [1, 93], which resembles the control of lipin-1 activity. Direct expression of lipin-1A and -1B in RH7777 cells also stimulates the production of apoB and increases its stability (submitted for publication) [96]. In order to assemble and secrete VLDL, a protein called microsomal triglyceride transfer protein (MTP) is required [104, 105]. We hypothesize that the change in lipin-1 expression may occur upstream of the change in MTP and ApoB expression. Moreover, lipin-1 itself may participate in controlling the expression level of MTP and ApoB, both of which are essential components for VLDL production and secretion. Therefore, it is interesting to study the time-course expression of both apoB and MTP mRNA in a time-course relative to the expression of the lipins. We expected to see an increase of lipin-1 preceding an increase of ApoB and MTP. If this holds true, it would also be interesting to overexpress lipin-1 in hepatocytes isolated from *fld* and control mice liver to determine if there is a change in the expression level of MTP and ApoB.

To summarize, GC increases the expression of lipin-1 mRNA in the primary culture of hepatocytes isolated from rat and mouse liver. cAMP synergizes this GC-induced increase in lipin-1 mRNA while insulin antagonizes it. No significant increase in the expression of lipin-2 and lipin-3 mRNA was observed when treated by GC. The increase in lipin-1 mRNA expression is responsible for the increases in lipin-1 protein level and PAP1 activity, which are found in conditions such as starvation and diabetes. The increase in PAP1 activity, found when there was a higher concentration of GC relative to insulin, enhances the liver capacity for hepatic glycerolipid synthesis and is reflected as steatosis in metabolic syndrome.

Figure 4.1. Mouse *Lpin1* promoter showing possible regulation sites for modulating GC-induced expression. The sequence is from -2040 to +70 kb. The search has been done using MATCH™ (Matrix Search for Transcription Factors Binding Sites) in TRANSFAC Professional®.

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APPENDIX

Glucocorticoids and cyclic AMP selectively increase hepatic lipin-1 expression, and insulin acts antagonistically

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Abstract **Glucocorticoids (GCs) increase hepatic phosphatidate phosphatase (PAP1) activity. This is important in enhancing the liver's capacity for storing fatty acids as triacylglycerols (TAGs) that can be used subsequently for β-oxidation or VLDL secretion. PAP1 catalyzes the conversion of phosphatidate to diacylglycerol, a key substrate for TAG and phospholipid biosynthesis. PAP1 enzymes in liver include lipin-1A and -1B (alternatively spliced isoforms) and two distinct gene products, lipin-2 and lipin-3. We determined the mechanisms by which the composite PAP1 activity is regulated using rat and mouse hepatocytes. Levels of lipin-1A and -1B mRNA were increased by dexamethasone (dex; a synthetic GC), and this resulted in increased lipin-1 synthesis, protein levels, and PAP1 activity. The stimulatory effect of dex on lipin-1 expression was enhanced by glucagon or cAMP and antagonized by insulin. Lipin-2 and lipin-3 mRNA were not increased by dex/cAMP, indicating that increased PAP1 activity is attributable specifically to enhanced lipin-1 expression. This work provides the first evidence for the differential regulation of lipin activities. Selective lipin-1 expression explains the GC and cAMP effects on increased hepatic PAP1 activity, which occurs in hepatic steatosis during starvation, diabetes, stress, and ethanol consumption.**—Manmontri, B., M. Sariahmetoglu, J. Donkor, M. B. Khalil, M. Sundaram, Z. Yao, K. Reue, R. Lehner, and D. N. Brindley. **Glucocorticoids and cyclic AMP selectively increase hepatic lipin-1 expression, and insulin acts antagonistically.** ***J. Lipid Res.*** **2008. 49: 1056–1067.**

Supplementary key words β-oxidation • diabetes • ethanol ingestion • fasting • glucagon • hypertriglyceridemia • steatosis • triacylglycerol synthesis

Mammalian phosphatidate phosphatase (PAP1) activity is Mg^{2+}-dependent and is inhibited by *N*-ethylmaleimide (1). These characteristics distinguish mammalian PAP1 from PAP2 activities that also convert phosphatidate (PA) to diacylglycerol (DAG). PAP2 is now commonly known as a family of lipid phosphate phosphatases (LPPs) that dephosphorylate a variety of lipid phosphate esters. The LPPs are mainly involved in regulating signal transduction (2). By contrast, PAP1 appears to be specific for PA as a substrate (3, 4) and is a required enzyme in the biosynthesis of triacylglycerol (TAG), phosphatidylcholine, and phosphatidylethanolamine (5).

Our previous work showed that injecting rats with cortisol or corticotropin produced marked increases in PAP1 activity in the liver (6, 7). Subsequent work with rat hepatocytes demonstrated that the glucocorticoid (GC) effect in increasing PAP1 activity was synergized by glucagon and inhibited by insulin (8, 9). We showed that these GC-induced increases in PAP1 activity provide the extra capacity for the liver to sequester excess FAs as TAG when these FAs are not immediately required for β-oxidation (5). The interaction of GC with insulin explains the diurnal rhythm of PAP1 activity in rat livers (10). The GC effect is also consistent with increases in hepatic PAP1 seen after sham operations or in liver remnants after partial hepatectomy (11), in starvation (6), diabetes (12), insulin resistance (13), and hypoxia (14), and in toxic conditions (5). Increases in hepatic PAP1 also occur in response to dietary modification in rodents, for instance, when glucose or starch is replaced by fructose, sorbitol, glycerol, or ethanol (15), and these effects are exaggerated by high-fat feeding (16). These changes in PAP1 are

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Abbreviations: CPTcAMP, 8-(4-chlorophenylthio) cyclic AMP; DAG, diacylglycerol; dex, dexamethasone; ER, endoplasmic reticulum; GC, glucocorticoid; LPP, lipid phosphate phosphatase; PA, phosphatidate; PAP1, phosphatidate phosphatase; PGC-1α, peroxisome proliferator-activated receptor-coactivator-1α; TAG, triacylglycerol.

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also associated with increased GC concentrations relative to insulin. PAP1 activity is also increased in the livers of baboons (17) and human alcoholics (18). The involvement of GC in ethanol-induced increases in PAP1 activity is confirmed because this is attenuated in adrenalectomized rats (19).

The physiological expression of PAP1 activity involves a FA-induced translocation of the reservoir of cytosolic PAP1 to become functional on membranes of the endoplasmic reticulum (ER), where PA is synthesized (20, 21). The activity of the membrane-bound PAP1 correlates closely with the conversion of PA to DAG and the synthesis of TAG and phosphatidylcholine in intact rat hepatocytes (21).

Further work in this area was severely hampered because of the inability of any group to purify or identify the structure of PAP1. This situation changed with a publication by Han, Wu, and Carman (4), who identified the yeast PAP1 (PAH1; previously known as SMP2) as an ortholog of mammalian lipin. They also showed that recombinant mammalian lipin-1 had PAP1 activity. Mammals express a family of lipins consisting of lipin-1A and its splice variant lipin-1B, plus lipin-2 and lipin-3 (22). In mature adipocytes, lipin-1A is preferentially located in the nucleus, whereas most of the lipin-1B is found in the cytosol (23). Our recent studies demonstrated that all of these lipins possess Mg^{2+}-dependent PAP1 activity and that they are expressed in a tissue-specific manner (3). For example, lipin-1 provides the majority, if not all, of the PAP1 activity in white and brown adipose tissue, skeletal muscle, and heart, whereas liver expresses lipin-1, -2, and -3 (3, 24). This observation explains why the *fld* mouse, which has a null mutation in the *Lpin1* gene, exhibits lipodystrophy, because lipin-1 is required for the development of mature adipocytes by regulating the expression of peroxisome proliferator-activated receptor γ (PPARγ) and TAG synthesis (25). The *fld* mouse also develops a fatty liver and hypertriglyceridemia in the preweaning period, which indicates an ability of the liver to synthesize and secrete TAG (26). This capacity in the *fld* mouse is explained by the expression of PAP1 activity through lipin-2 and -3 (3, 24). In fact, livers from *fld* mice show normal PAP1 activity and increased lipin-3 mRNA levels, presumably as an adaptive response to the lack of lipin-1 (3). In addition to controlling TAG synthesis, lipin-1 increases the capacity of the liver for β-oxidation in fasting by facilitating transcriptional regulation by peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) and PPARα (27).

The discovery that the liver expresses lipin-1A, -1B, -2, and -3 (3) provokes the question of which lipins respond to hormonal regulation to explain the observed physiological changes in the composite PAP1 activity. Answering this question and describing the mechanisms that control the expression of the different lipins are essential to establishing their functions in hepatic metabolism and for understanding the hormonal regulation of their expression. To investigate this, we compared the responses of primary cultures of rat and mouse hepatocytes over a time course after treatment with hormones and 8-(4-chlorophenylthio) cyclic AMP (CPTcAMP). The results show that the members of the lipin family were differentially regulated by dexamethasone (dex), glucagon, and insulin. Dex with glucagon or CPTcAMP markedly increased total PAP1 activity, and this effect was accounted for by the increased synthesis of lipin-1. Insulin attenuated the dex- + CPTcAMP-induced increases in lipin-1 synthesis. These results provide the first evidence for the differential regulation of the activity of different lipins in the liver. They help to explain how the composite changes in PAP1 (lipin) activity may coordinate increased TAG synthesis, β-oxidation, and VLDL secretion in conditions of starvation, metabolic stress, insulin resistance, and diabetes.

METHODS

Materials

Dex, CPTcAMP, insulin, and glucagon was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies against lipin-1 were prepared in accordance with University of Ottawa Heart Institute regulations relating to Animal Care Procedures using the peptide SKTDSPSRKKDKRSRHLGADG essentially as described previously (28). The antibody was used at a dilution of 1:500. Mouse monoclonal antibody for the V5 tag and GAPDH were purchased from Invitrogen and Sigma and used at dilutions of 1:1,000 and 1:5,000, respectively. Secondary antibodies were IRDye 800 goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) and goat anti-mouse IgG conjugated to Alexa Fluor 680 (Molecular Probes, Eugene, OR).

Preparation and culture of hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats (200–340 g) or C57BL/6J mice (22.5–32 g) as described previously (29). They were plated onto collagen-coated dishes in DMEM containing 15% serum in an atmosphere of 95% air and 5% CO_2 for 45–90 min to allow attachment. The medium was changed to remove nonviable cells, and the hepatocytes were incubated for a further 4 h to allow them to spread. The medium was then changed, and the hepatocytes were incubated for different times in serum-free medium containing 0.1% BSA with the addition of hormones or agonists as indicated. All incubations contained 0.5% DMSO, which was used as a vehicle for dex.

Gene expression analysis in fasting/refeeding conditions

Livers were harvested from 16 week old female C57BL/6J mice after fasting for 16 h (fasted samples) or fasting for 16 h followed by refeeding for 4 h (refed samples). Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) and cDNA synthesized from 2 μg of RNA using the Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed with the iCycler (Bio-Rad, Hercules, CA) using SYBR Green PCR reagents (Qiagen) as described previously (25). Gene expression was normalized to levels of β_2-microglobin and 18S rRNA. Primers used for this fasting study are listed in Table 1.

RNA quantitation for hepatocyte samples by real-time RT-PCR

RNA was collected using the RNAqueous Kit (Ambion, Inc., Austin, TX) according to the manufacturer's directions. Reverse

TABLE 1. Oligonucleotide primers used for real-time RT-PCR in fasting experiments with mice

Protein	Forward Primer	Reverse Primer	Reference
Mouse 18s rRNA	accgcagctaggaataatgga	gcctcagttccgaaaacca	
Mouse β_2-microglobin	gtctttcagcaaggactggtc	caaatgcggcatcttcaaacc	48
Mouse lipin-1A	ggtccccagccccagtcctt	gcagcctgtggcaattca	23
Mouse lipin-1B	cagcctggtagattgccaga	gcagcctgtggcaattca	23
Mouse lipin-2	agttgacccatcaccgtag	cccaaagcatcagacttggt	3
Mouse lipin-3	tggaattgggatgacaaggt	cactgcaagtaccccttggt	3
Mouse PGC-1α	ctcacagagacactggacagt	tgtagctgagctgagtgttgg	
Mouse PPARα	aatgcaattcgctttggaag	ggccttgacctgttcatgt	48

PGC-1α, peroxisome proliferator-activated receptor-coactivator-1α; PPARα, peroxisome proliferator-activated receptor α. Where not referenced, primers were designed by Primer3 or Primer Express version 2.0 software using default parameters.

transcription was performed using SuperScript II, random primers, and RNaseOUT according to instructions from the supplier (Invitrogen). PCR was performed on an iCycler (Bio-Rad) using SYBR Green PCR reagents (Applied Biosystems, Foster City, CA). Primer sequences for PCR are listed in **Table 2**. Gene expression was normalized to the housekeeping genes cyclophilin A and GAPDH. In initial experiments, the relative changes in mRNA expression for the lipins were essentially the same when expressed relative to both reference mRNAs; therefore, we routinely expressed results relative to cyclophilin A mRNA.

Measurement of PAP1 activity

Hepatocytes were lysed in 0.25 M sucrose containing 2 mM dithiothreitol, 0.15% Tween 20, and a protease inhibitor cocktail (Sigma). We developed the following assay specifically to give accurate measurements of relative PAP1 activity in tissue and cell homogenates, which are able to metabolize PA by several different routes (3, 30). We chose to measure the formation of DAG from PA labeled with [^{3}H]palmitate (3, 30) in preference to the release of water-soluble ^{32}P from [^{32}P]PA. In liver or hepatocyte homogenates, glycerol-^{32}P can be produced by phospholipase A action, and this product is further converted to inorganic ^{32}P (31). Thus, this latter assay with crude enzyme preparations has to be used with care to ensure that the measured ^{32}P is only produced by PAP activity (32). We also mixed the PA in the molar ratio of 3:2 with nonradioactive PC, because this form of the substrate maximizes PAP1 activity relative to that of PAP2 (1). In our assays, ~90% of the PAP activity is from PAP1 (3). Had we used Triton X-100 to solubilize the PA, this would extract lipids, hydrophobic proteins, and amphiphilic proteins from the homogenates, and the advantage of this "defined substrate" would immediately be lost. Moreover, the use of PA dissolved in micelles of Triton X-100 favors PAP2 activity relative to that of PAP1 (1). It is important in assays for PAP1 to eliminate the contribution from PAP2, and this is best done in these mammalian systems by inhibiting PAP1 activity with *N*ethylmaleimide rather than by trying to eliminate the effects of endogenous Mg^{2+} (30, 33). This value was then subtracted from the total activity to give PAP1 activity. Thus, maximizing the PAP1 activity relative to PAP2 makes this correction smaller and leads to greater precision in estimating PAP1 activity in tissue and cell homogenates. We also discovered that adding Tween-20 to the homogenates stabilizes and increases PAP1 activity (34). In addition, Tween-20 appears to minimize the breakdown of the DAG product by lipases in the assay, making the use of tetrahydrolipstatin to inhibit this activity unnecessary (3).

Briefly, samples were assayed in 100 mM Tris/maleate buffer, pH 6.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mg/ml FA-poor BSA, 0.6 mM PA labeled with [^{3}H]palmitate (~1×10^5 dpm/assay), and 0.4 mM phosphatidylcholine. The [^{3}H]DAG product was purified using alumina and then quantitated by scintillation counting (33). Parallel incubations were performed in the presence of excess (8 mM) *N*ethylmaleimide to inhibit PAP1 and to compensate for any PAP2 (LPP) activity in this assay (30). The amount of cell homogenate was adjusted so that the formation of DAG consumed <15% of the PA added. Reaction rates were measured at three different protein concentrations to ensure the proportionality of the assay

TABLE 2. Oligonucleotide primers used for real-time RT-PCR in hepatocyte experiments

Protein	Forward Primer	Reverse Primer	Reference
Cyclophilin A	caccgtgttcttcgacatcac	ccagtgctcagagctcgaaag	49
Mouse GAPDH	tgtgtccgtcgtggatctga	cctgcttcaccaccttcttga	
Mouse lipin-1A	gcctgctcgtgaatcctct	cgatgcatccgacagcgt	23
Mouse lipin-1B	cagcctggtagattgccaga	gcagcctgtggcaattca	23
Rat lipin-1	tcactaccagtaccaggge	tgagtccaatccttttccag	
Rat lipin-1B	agcagcctggtagattgtca	taagggctggagtcttttcat	
Rat and mouse lipin-2	tagatgcagaccctgttccc	ctggtgctggcttcttttgt	
Rat and mouse lipin-3	aaagactggacacaccaggg	tgctggatatcactcaggca	
Mouse PGC-1α	ggcacgcagcctctattca	cgacacggagagttaaaggaaga	
Mouse PPARα	actacggagttcacgcatgtg	ttgtcgtacaccagcttcagc	50
Rat PGC-1α	cacaacgcggacagaactga	ccgcagatttacggtgcatt	
Rat PPARα	tggagtccacgcatgtgaag	cgccagcttttagccgaatag	

Where not referenced, primers were designed by Primer3 or Primer Express version 2.0 software using default parameters.

for each sample, so that the relative rates of PAP1 activity can be calculated.

Expression of recombinant lipins

Lipin-1A, -1B, -2, and -3 that were tagged with a V5 epitope were expressed in HEK 293 cells as described previously (3), and these were used to test the specificity of the lipin-1 antibody.

Western blot analysis

Protein concentrations in cell lysates were determined using the Bradford protein assay (Bio-Rad). Identical amounts of protein (100 μg) were mixed with a commercial loading buffer (Invitrogen), and proteins were separated by SDS-PAGE (35) using 8% gels. The proteins were transferred onto nitrocellulose membranes (Bio-Rad), which were blocked with Odyssey® blocking buffer (Li-Cor Biosciences, Lincoln, NE). Membranes were then incubated with rabbit antibodies for lipin-1 or mouse monoclonal antibody for GAPDH or V5. The membranes were then washed and incubated with IRDye 800 goat anti-rabbit IgG and goat anti-mouse IgG conjugated to Alexa Fluor 680, respectively. Simultaneous images obtained at 700 and 800 nm were quantified using the Odyssey® Imager System (Li-Cor).

Statistical analysis

The significance of differences among treatments was analyzed using a Newman-Keuls post hoc test for a one-way ANOVA or a Bonferroni test with a two-way ANOVA.

RESULTS

To investigate how the expression of different lipins is regulated in the liver, we used primary cultures of mouse hepatocytes to relate this work to various mouse genetic models that exist or will be created. We also used rat hepatocytes, because our earlier work showing marked hormone-induced changes in PAP1 activity was performed with the rat. We incubated the hepatocytes for various times with an optimum (100 nM) concentration (9) of dex, a synthetic GC, because the natural corticosterone is efficiently degraded by hepatocytes (9). We also determined interactions of dex with glucagon, CPTcAMP, or insulin.

Hormonal regulation of the expression of mRNA for lipin-1A and -1B

In mouse hepatocytes, the relative mRNA levels for lipin-1A and -1B were increased by dex and reached a peak after ~4 h (Fig. 1A, C). This dex effect was amplified by the presence of CPTcAMP (Fig. 1A, C) or by glucagon in the case of lipin-1B (Fig. 2A). CPTcAMP or glucagon alone had no significant effect in increasing the mRNA for lipin-1A or -1B compared with the untreated (control) incubations (Figs. 1A, C, 2A). Insulin alone had no significant effect on the relative mRNA concentrations for lipin-1A or -1B compared with the nontreated control. However, it attenuated the effects of dex alone and dex with CPTcAMP or glucagon (Figs. 1B, D, 2A).

For work with rat hepatocytes, we chose to use primers for total lipin-1 and lipin-1B, because at the beginning of this work we were uncertain about the nucleotide sequences for these lipins. The levels of mRNA for lipin-1 and -1B in the nontreated controls declined as the incubation proceeded (Fig. 3A, C). Dex increased the levels of these mRNAs relative to the value at time 0 of incubation and even more so compared with the nontreated control at the equivalent time. Maximum increases were obtained after 4–8 h of incubation. CPTcAMP or glucagon alone had no significant effect on the levels of mRNA for lipin-1 and -1B (Figs. 2B, 3A, B). Although CPTcAMP or glucagon appeared to have a slight effect in augmenting the dex-induced increase in the levels of mRNA for total lipin-1 and -1B, this only reached statistical significance for lipin-1 and -1B at the 8 and 4 h, respectively. Insulin alone had no sig-

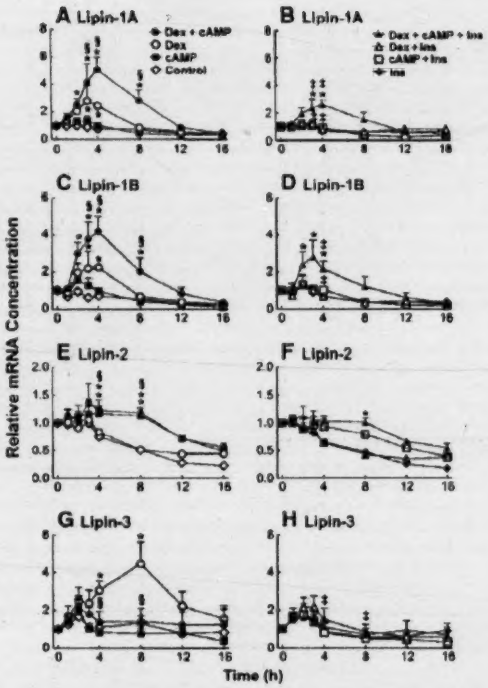

Fig. 1. Interaction of dexamethasone (dex), cAMP, and insulin in controlling mRNA expression for lipin in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM 8-(4-chlorophenylthio) cyclic AMP (CPTcAMP; cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for the different lipins were measured (A–H) by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for 3–15 independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; ‡ dex alone different from dex + CPTcAMP treatment; † incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

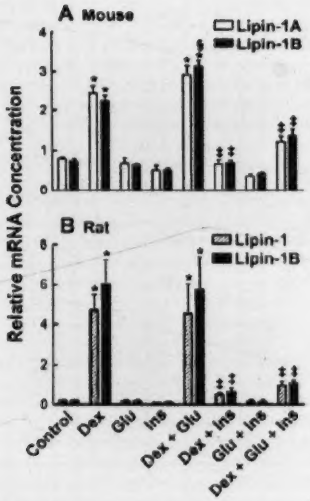

Fig. 2. Interaction of dex, glucagon, and insulin in controlling mRNA expression for lipin-1 in mouse and rat hepatocytes. Mouse (A) and rat (B) hepatocytes were incubated for 4 and 8 h, respectively, with 100 nM dex (Dex), 10 nM glucagon (Glu), and 100 nM insulin (Ins) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are means ± SEM for 3–15 independent experiments for the mouse and for 3 to 8 experiments for the rat. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; $ dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + glucagon.

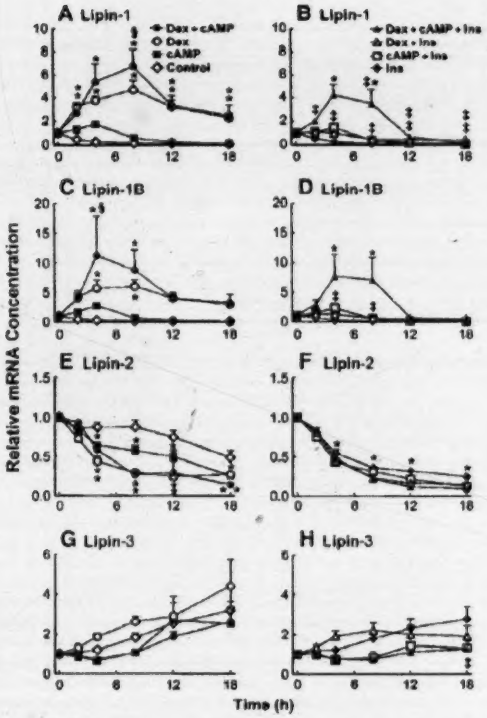

Fig. 3. Interaction of dex, cAMP, and insulin in controlling mRNA expression for lipin in rat hepatocytes. Rat hepatocytes were incubated for various times with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured (A–H) by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three to eight independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; $ dex alone different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

nificant effect on mRNA levels for lipin-1 or -1B, but it decreased the effects of dex alone, or dex with CPTcAMP, or glucagon (Figs. 2B, 3B, D).

Actinomycin D blocked the dex effect on the levels of mRNA for lipin-1A or -1B in mouse hepatocytes (Fig. 4) and for lipin-1 and -1B in rat hepatocytes (results not shown). This demonstrates that the increase in mRNA depends on increased transcription. Conversely, the dex-induced expression of mRNA for lipin-1 and -1B in rat hepatocytes was not decreased by the presence of cycloheximide, an inhibitor of protein synthesis (results not shown). With mouse hepatocytes, cycloheximide decreased the relative dex-induced increase in mRNA for lipin-1A and -1B (Fig. 4D, E).

Hormonal regulation of expression of mRNA for lipin-2

For mouse hepatocytes, no treatment that we used increased lipin-2 mRNA levels (Fig. 1E, F). In fact, the expression of mRNA for lipin-2 decreased during the incubation period in the nontreated controls and in dex- or insulin-treated hepatocytes. The presence of CPTcAMP alone, or in combination with dex, or insulin maintained the mRNA at the starting level for ~8 h. For rat hepatocytes, lipin-2 mRNA levels decreased over the period of incubation under all conditions (Fig. 3E, F).

Hormonal regulation of expression of mRNA for lipin-3

Treatment of mouse hepatocytes with dex increased the relative lipin-3 mRNA concentrations by ~4-fold after 8 h (Fig. 1G). CPTcAMP or insulin alone had no significant effect relative to the nontreated control, but both decreased the dex-induced increase in lipin-3 mRNA (Fig. 1G, H). Treatment with actinomycin D or cycloheximide also blocked the dex-induced increase in mRNA (Fig. 4C, F). These inhibitors also attenuated the expression of lipin-3 mRNA in the nontreated control hepatocytes after 8 h (Fig. 4C, F) or 4 h (results not

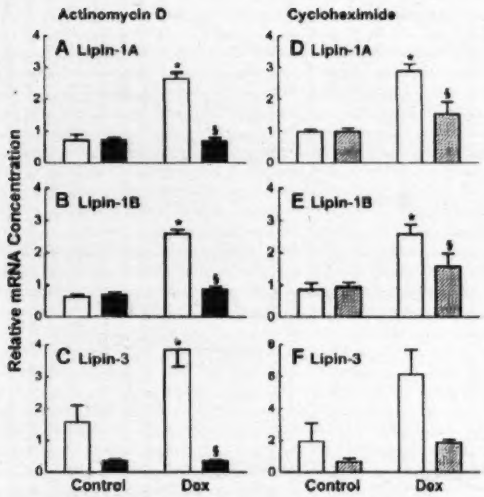

Fig. 4. Effects of actinomycin D and cycloheximide on the dex-induced increase in mRNA for lipin-1A, -1B, and -3. Mouse hepatocytes were treated with or without dex (Dex) in the presence or absence of 10 μg/ml actinomycin D (cells were also preincubated for 30 min with actinomycin D) (A–C) or 5 μg/ml cycloheximide (D–F). To measure mRNA production for lipin-1A and -1B or lipin-3, the hepatocytes were incubated for 4 or 8 h, respectively, based upon the time required to achieve optimum stimulation of mRNA production as show in Fig. 1. White columns show values for incubations in the absence of inhibitor, whereas black and hatched columns indicate the presence of actinomycin D or cycloheximide, respectively. Results are means ± SEM for three independent experiments, except for F, where means ± ranges are shown for two experiments. The significance of the differences ($P < 0.05$), as evaluated with a Student's *t*-test, is indicated as follows: * dex treatment different from the untreated control value; $ the actinomycin D or cycloheximide result is different from the equivalent incubation without these inhibitors.

shown). One explanation for these results is that the transcription of lipin-3 mRNA in mouse hepatocytes is dependent on the rapid synthesis of an unidentified protein.

In rat hepatocytes, none of the hormonal treatments changed lipin-3 mRNA levels relative to the nontreated control (Fig. 3G, H). There appeared to be a gradual increase in lipin-3 mRNA levels during the 18 h of incubation even in the nontreated control (Fig. 3G, H). This effect was blocked by actinomycin D or cycloheximide (results not shown).

Relationship of the expression of mRNA for peroxisome proliferator-activated receptor-coactivator-1α and PPARα to that of lipin-1

Full induction of hepatic lipin-1 expression under conditions such as fasting requires the presence of peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α), and lipin-1 also interacts physically with both PGC-1α and PPARα (27). To understand whether the effects of dex, CPTcAMP, and insulin on the expression of mRNA for lipin-1A or -1B depend upon prior changes in the transcription for PGC-1α and PPARα, we determined the time course and hormonal requirements for expression.

In mouse hepatocytes, the maximum effect of CPTcAMP in increasing mRNA expression for PGC-1α was achieved by 4 h. Dex alone had no significant effect on the expression of mRNA for PGC-1α, but it synergized the action of CPTcAMP (**Fig. 5A**). Insulin did not significantly affect these actions of CPTcAMP and dex + CPTcAMP (Fig. 5B).

Dex produced maximum increases in mRNA expression for PPARα by 4 h (Fig. 5C). CPTcAMP alone did not change the mRNA expression, but there was an indication that it might have delayed the maximum expression of PPARα. Insulin did not modify the effect of dex or dex + CPTcAMP significantly (Fig. 5D).

In rat hepatocytes, CPTcAMP increased mRNA expression for PGC-1α, especially in the presence of dex. The maximum increase occurred after ~8 h (**Fig. 6A, B**). Insulin had no significant effect in inhibiting these actions of CPTcAMP and dex + CPTcAMP.

Dex alone increased the mRNA for PPARα after 8 h (Fig. 6C, D), but in contrast to mouse hepatocytes, CPTcAMP partly attenuated this effect. There was also a pronounced effect of insulin in attenuating the dex-induced increase in mRNA for PPARα, which was not seen in mouse hepatocytes.

These results from mouse and rat hepatocytes show that the induction of mRNA expression for PGC-1α and PPARα by CPTcAMP and dex, respectively, occurs at the same time rather than preceding that for lipin-1A and -1B. Dex alone does not increase the expression of PGC-1α mRNA; therefore, the dex-induced production of lipin-1 mRNA cannot rely on an indirect effect through PGC-1α production. Also, insulin does not block the increase in mRNA for PGC-1α in mouse or rat hepatocytes. Therefore, the insulin effect in decreasing the production of mRNA for lipin-1A and -1B cannot be mediated by decreasing the transcription regulation of *Lpin1* by regulating PGC-1α expression.

Dex and CPTcAMP increase PAP1 activity in mouse and rat hepatocytes, and insulin partially blocks these effects

These experiments were designed to relate the changes in the lipin mRNA concentrations to those in PAP1 activity. Dex increased total PAP1 activity in mouse hepatocytes by ~1.8-fold after 8 h of incubation compared with the activity at the beginning of the incubation (**Fig. 7A**). The dex-induced increase in PAP1 activity was enhanced to >2-fold when CPTcAMP was added together with dex (Fig. 7A). These increases in PAP1 activity remained until at least 16 h. When added alone, CPTcAMP had no significant effect on PAP1 activity. Insulin alone also had no significant effect on PAP1 activity, but it partially reversed the increases produced by dex or dex + CPTcAMP (Fig. 7B).

In rat hepatocytes, dex increased PAP1 activity by ~2.5-fold after incubation for 12 or 18 h compared with

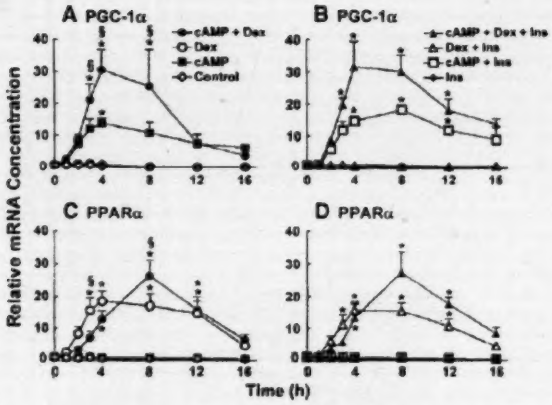

Fig. 5. Interaction of dex, cAMP, and insulin in controlling mRNA expression for peroxisome proliferator-activated receptor α (PPARα) and peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (A, B) and PPARα (C, D) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment.

the activity at the beginning of the incubation (Fig. 7C). CPTcAMP did not increase PAP1 activity, but it prevented the decline of up to 70% in activity that occurred after 18 h in the untreated controls or in insulin-treated cells (Fig. 7C, D). These results are compatible with the known stability of PAP1 activity in rat hepatocytes. PAP1 activity in untreated or insulin-treated cells has a half-life of 5–7 h, and this is increased to 12 h by glucagon (through cAMP) (36). Adding CPTcAMP together with dex in the present experiments produced a synergistic effect, and increases of >4-fold in PAP1 activity were obtained after 12 and 18 h (Fig. 7C). Insulin attenuated the actions of dex alone or dex + CPTcAMP (Fig. 7D).

Dex and CPTcAMP increase lipin-1 synthesis and protein levels, and insulin attenuates these effects

The purpose of these experiments was to investigate whether the dex-induced increases in lipin-1 mRNA expression result in increased expression of lipin-1 protein. To do this, we first validated the quality of the antibody that we used. **Figure 8A** shows the results of a Western blot for recombinant lipin-1A, -1B, -2, and -3 containing a V5 tag that were individually expressed in HEK 293 cells (3). The blots were probed simultaneously with mouse monoclonal anti-V5 antibody and rabbit polyclonal anti-lipin-1 antibody. The results show coincident detection of lipin-1A and -1B by the anti-lipin-1 antibody and V5 antibodies. There was no cross-reactivity of the anti-lipin-1 antibody with lipin-2 and -3. The second test was to compare the Western blots from adipose tissue of wild-type mice and *fld* mice, which are deficient in lipin-1 (3). The rabbit antibody detected lipin-1 in the sample from a wild-type mouse, but there was no response with the same amount of protein from the *fld* mouse (Fig. 8B). These results provide evidence that the lipin-1 antibody can specifically detect lipin-1A and -1B.

Figure 8B also shows the Western blots for lipin-1 from mouse and rat hepatocytes that were incubated for 8 and 12 h, respectively, with the various combinations of hormones and CPTcAMP based upon the

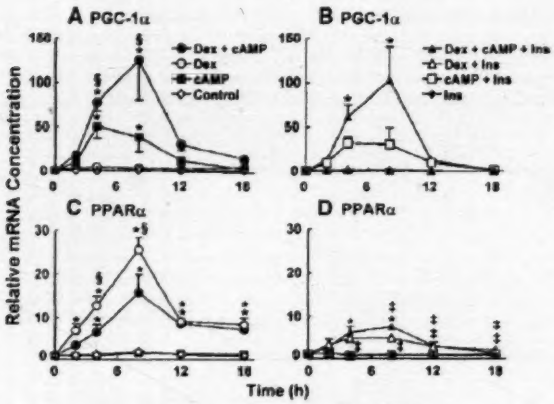

Fig. 6. Interaction of dex, cAMP, and insulin in controlling mRNA expression for PPARα and PGC-1α in rat hepatocytes. Rat hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (A, B) and PPARα (C, D) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

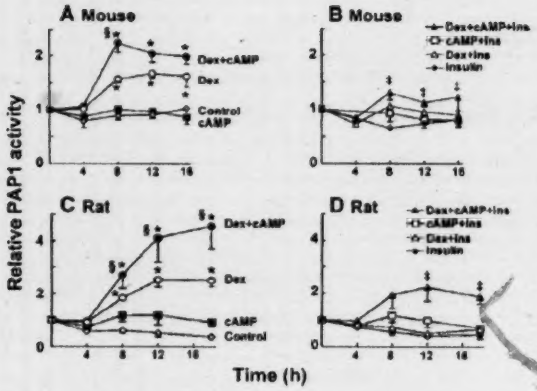

Fig. 7. Interaction of dex, glucagon, and insulin in controlling phosphatidate phosphatase (PAP1) activity in mouse and rat hepatocytes. Mouse (A, B) and rat (C, D) hepatocytes were incubated for various times with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. The average PAP1 specific activities at the beginning of the incubation were 32 ± 15 (n = 7) and 36 ± 12 (n = 5) nmol diacylglycerol produced per min per mg protein for mouse and rat hepatocytes, respectively. The results are expressed relative to the initial untreated value, which was normalized to 1. Results are means ± SEM for three to nine independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

changes in PAP1 activity from Fig. 7. The same amount of protein from these samples was loaded onto each lane, and GAPDH was used as an additional loading control (Fig. 8B). The results for lipin-1 were expressed relative to GAPDH, and the average results are illustrated in Fig. 8C. Lipin-1 was detected as multiple bands, which probably represent different phosphorylation states (24). Incubation of mouse hepatocytes with dex increased the relative lipin-1 protein levels by ~2.5-fold, and this was further increased to ~4.5-fold when CPTcAMP was

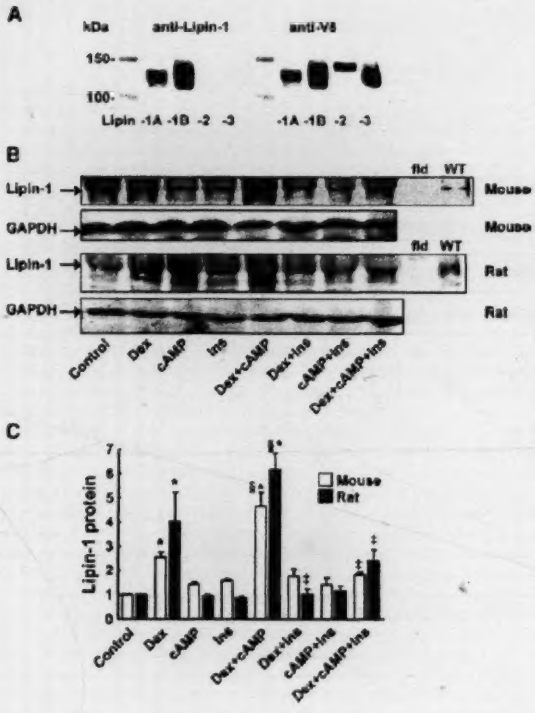

Fig. 8. Interaction of dex, glucagon, and insulin in controlling lipin-1 expression in mouse and rat hepatocytes. A: Western blots for different V5-tagged lipins that were detected simultaneously with a rabbit polyclonal anti-lipin-1 antibody and a mouse monoclonal anti-V5 antibody. B: Representative Western blots for mouse and rat hepatocytes that were incubated for 8 and 12 h, respectively, with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. A marker for lipin-1 is shown on the right side, where adipose tissue extracts from wild-type (WT) and *fld* mice were used. The lower Western blots are for GAPDH, which was used as a loading control. C: Means ± SEM for the relative expression of lipin-1 after normalization against GAPDH for each treatment. Results are for three to four independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

present (Fig. 8C). CPTcAMP had no significant effect on lipin-1 protein levels when added alone. Insulin alone had no effect on lipin-1 protein levels, but it attenuated the effects of dex and dex + CPTcAMP. The results are expressed relative to the nontreated control at the 8 h time point. This value was ~40% of that at the beginning of the incubation.

Incubation of rat hepatocytes with dex increased the relative lipin-1 protein levels by ~4-fold, and this increased to ~6-fold when CPTcAMP was also present (Fig. 8B, C). Incubation with CPTcAMP alone had no significant effect on lipin-1 protein levels. Insulin alone also had no significant effect, but it blocked the effects of dex and attenuated the actions of dex + CPTcAMP. Lipin-1 protein levels decreased by ~50% in the nontreated controls after the 12 h incubation (results not shown). Therefore, the relative increases in the expression of lipin-1 with dex and dex + CPTcAMP that are shown in Fig. 8B would be approximately halved if expressed relative to the value at the beginning of the incubation. By comparison, the results for the mouse would only be affected by ~10%.

The dex-induced increases in lipin-1 protein levels in rat and mouse hepatocytes were blocked by actinomycin D or cycloheximide, and this was reflected in the lack of increase in PAP1 activity (Figs. 9A, B). These combined results demonstrate that the dex-induced increase in lipin-1 and PAP1 activity depends upon increases in both transcription and translation.

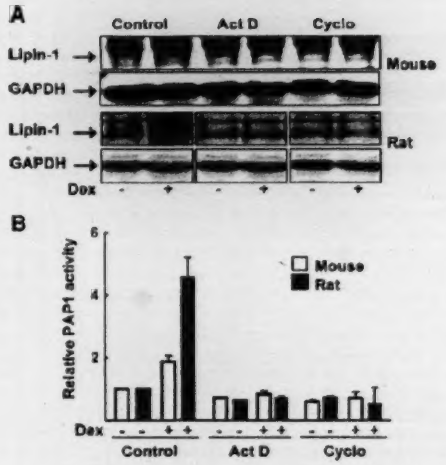

Fig. 9. Actinomycin D and cycloheximide block the dex-induced expression of lipin-1 and PAP1 activity. Mouse and rat hepatocytes were preincubated with or without 10 μg/ml actinomycin D (Act D) or 5 μg/ml cycloheximide (Cyclo) for 30 min. The inhibitors were maintained in the subsequent incubations in the presence or absence of 100 nM dex, which for mouse and rat hepatocytes were 8 and 12 h, respectively. These times were based upon the results in Fig. 4. A: Representative Western blots. B: PAP1 activity relative to the equivalent incubation in the absence of inhibitors or dex. Results are means ± SEM for three independent experiments with mouse hepatocytes and means ± ranges for two experiments with rat hepatocytes.

Effects of fasting mice on the expression of mRNA for different lipins in the liver

The next series of experiments were performed to relate our observations with hepatocytes to the effects of fasting mice on the expression of mRNA for different lipins, PPARα, and PGC-1α in the liver. mRNA concentrations for lipin-1A, -1B, and -2 were increased, on average, by 4.3-, 3.2-, and 2.4-fold respectively, but there was no significant change in mRNA for lipin-3 (Fig. 10). Fasting also increased the mRNA for PGC-1α by 2.4-fold, as expected (37), although the apparent increase for PPARα did not reach statistical significance.

DISCUSSION

The recent identification of lipin proteins as PAP1 enzymes made it possible to investigate the molecular basis for the modulation of PAP1 activity that occurs in various physiological and pathological conditions. The liver expresses lipin-1A, -1B, -2, and -3; therefore, it was essential to elucidate how each lipin responds to GC, glucagon, and insulin to understand the functions of individual lipins and the reason for changes in the composite PAP1 activity. Our present experiments with rat and mouse hepatocytes demonstrate a level of hormonal control that is preserved between these rodent species. The lipin-1A and -1B isoforms, alternative splice variants of the *Lpin1* gene, are induced at the transcriptional level by the action of dex. This dex action, especially in mouse hepatocytes, was synergized by glucagon, whereas insulin attenuated this effect. Insulin can block the effects of glucagon by stimulating phosphodiesterase activity and diminishing the increase in cAMP. However, we also used CPTcAMP, which is not readily degraded by phosphodi-

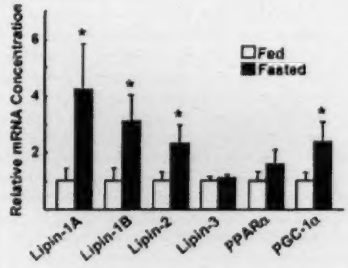

Fig. 10. Effects of fasting on mRNA expression for the lipins, PPARα, and PGC-1α in mouse liver. mRNA concentrations were measured by real-time PCR in livers from C57BL/6J mice that were fasted for 16 h (fasted samples) or fasted for 16 h and refed for 4 h (fed samples). Results are means ± SD for three mice in each group, and significant differences compared with the fed values are indicated (* $P < 0.05$).

esterase (38), and observed the same insulin attenuation of *Lpin1* transcription. This result indicates that insulin exerts a more direct effect on lipin-1 expression.

There is no evidence that the hormonal combinations we used regulate the relative expression of the lipin-1 splice isoforms in hepatocytes. The dex-induced increases in PAP1 activity depended upon transcription of the *Lpin1* gene and increased lipin-1 mRNA, which was followed by increases in lipin-1 synthesis and protein expression. This conclusion is supported by the effects of actinomycin D and cycloheximide, which blocked the increase in these parameters.

Our results demonstrate that the expression of lipin-2 and -3 is regulated in a distinct manner from that of lipin-1. Incubation of mouse and rat hepatocytes with dex resulted in a time-dependent decrease in lipin-2 mRNA. Dex did produce small increases in lipin-3 mRNA in mouse hepatocytes, but this effect was blocked by CPT-cAMP. In rat hepatocytes, there was no significant effect of dex or dex + CPTcAMP on lipin-3 mRNA expression relative to nontreated controls. Therefore, increased transcription of the *Lpin2* and *Lpin3* genes did not contribute to the dex + CPTcAMP-induced increase in PAP1 activity in isolated rat or mouse hepatocytes, which depended upon increased transcription and translation. At present, we are unable to perform satisfactory Western blot analysis for lipin-2 and -3 because we lack convincing evidence that the antibodies we possess selectively identify lipin-2 or -3 in hepatocyte extracts.

Our conclusions that the dex- and dex + cAMP-induced increases in PAP1 activity are accounted for by increased lipin-1 expression are strongly supported by studies in vivo. Livers of fasted *fld* mice, which do not express lipin-1, do not show increases in PAP1 activity compared with ~2-fold increases in control mice (24). Lipin-1 transcription and lipin-1 protein levels in the livers of wild-type mice are increased after fasting or dex injection (27). In our experiments with fasted mice, we also observed increases in mRNA for lipin-1A, -1B, and PGC-1α but not for lipin-3. Surprisingly, the relative mRNA concentration for lipin-2 was doubled in the livers of fasted mice. This is unlikely to have occurred by GC action, based upon our hepatocyte work. It could have resulted from a cAMP effect, because this second messenger appeared to increase the relative concentration of lipin-2 mRNA in mouse hepatocytes relative to the nontreated, or dex-treated, cells. However, the physiological significance of the increased lipin-2 mRNA in fasted mice is uncertain, because of the lack of increase in hepatic PAP1 activity in *fld* mice after fasting (24). The combined results from experiments in vivo strongly support our conclusion that increased lipin-1 expression accounts for the increased PAP1 activity that occurs when there is increased GC action compared with insulin.

However, there could be other factors that contribute to the observed increase in PAP1 activity. First, we established that glucagon, through cAMP, increases the half-life of GC-induced PAP1 activity (which we now ascribe to lipin-1) from ~7 to 12 h (36). This could contribute to the increased expression of the lipin-1 protein that is produced by dex + CPTcAMP. Second, CPTcAMP could affect PAP1 activity through its phosphorylation, and we provided indirect evidence for this before the lipins were discovered (8, 39). Direct evidence for the phosphorylation of yeast PAP1 (PAH1) has now been obtained (40). In adipose tissue, the level of lipin-1 phosphorylation is controlled by the balance of signaling from insulin versus cAMP (24, 28). These changes in lipin-1 phosphorylation were not reflected in changes in PAP1 activity as measured in vitro (24), although treatment of liver cytosol with phosphatases did decrease total PAP1 activity in earlier work (39). The major effect of lipin-1 phosphorylation appears to be to regulate its subcellular distribution (24) and its physiological expression (5).

A third explanation for the regulation of lipin-1 and PAP1 activity is through interaction with other proteins, including PGC-1α and PPARα (27). Not only does lipin-1 amplify signaling by PGC-1α and PPARα, but PGC-1α is required as a coamplifier of lipin-1 expression in the liver. Thus, induction of lipin-1 expression in fasted or in dex-treated mice is partially attenuated when PGC-1α is totally deficient, indicating a partial dependence on PGC-1α. Hepatic lipin-1 expression is also increased in type 1 and type 2 diabetes, conditions in which PGC-1α expression is increased (27). Our results showing that cycloheximide partially inhibits the dex-induced production of lipin-1 mRNA in mouse hepatocytes indicate that the induced expression of cotranscriptional regulators, including PGC-1α, may be required for full lipin-1 expression. In rat hepatocytes, cycloheximide produced no significant decrease in lipin-1 mRNA expression. This could indicate that the endogenous levels of cotranscriptional regulators for lipin-1 mRNA production in rat hepatocytes are sufficient to sustain high levels of transcriptional induction. Significantly, CPTcAMP had only a marginal effect in increasing the dex-induced production of lipin-1 mRNA in rat hepatocytes, whereas the effect was more marked in mouse hepatocytes.

As expected from previous work (37, 41), mRNA for PGC-1α was increased by CTPcAMP in mouse and rat hepatocytes (Figs. 5, 6). Dex alone had no significant effect on the relative mRNA for PGC-1α, but it strongly synergized the CPTcAMP action. By contrast, dex was effective in increasing mRNA expression for PPARα, as expected (42), and there was little if any effect of CPTcAMP. These combined observations are compatible with the increased expression of PGC-1α and PPARα in starvation and diabetes (37, 41). Although insulin decreases the signaling effects of PGC-1α in vivo (37, 41), there was no significant effect of insulin in directly decreasing the expression of PGC-1α mRNA in mouse and rat hepatocytes. Our results for PGC-1α agree with previous work with mouse hepatocytes (41). Although lipin-1 expression depends partly on the presence of PGC-1α (27), the effects of dex + CPTcAMP in increasing the mRNA for PGC-1α and PPARα do not precede the maximum expression of mRNA for lipin-1A or -1B. Also, the inhibition of lipin-1 mRNA expression by insulin probably results from a direct action

on transcription of the *Lpin1* gene, because insulin did not block the stimulated increase in mRNA for PGC-1α. In the case of PPARα, we did see an attenuation of the dex induction in mRNA concentrations in rat hepatocytes, but this insulin effect was not observed with the mouse hepatocytes.

In liver, the interaction of lipin-1 with PGC-1α and PPARα promotes the transcriptional regulation of enzymes involved in β-oxidation, and this is also a response to starvation and diabetes (27). We proposed that the GC-induced increase in PAP1 activity could be an adaptive response to protect the liver against an increased FA load and lipotoxicity in starvation and diabetes (5, 8). However, the accumulation of TAG in the liver could also be lipotoxic (43). We now show that lipin-1 specifically is responsible for the GC-induced increase in PAP1 activity, which we previously showed to result in increased hepatic glycerolipid synthesis (7). As FA concentrations increase and exceed the capacity for β-oxidation, PAP1 translocates to ER membranes to facilitate the storage of the excess FA as TAG in fat droplets, resulting in steatosis (5). The FA-induced translocation of lipin-1 occurs in adipocytes (24). TAG synthesis in the liver should not be viewed simply as antagonistic to β-oxidation. It is a companion pathway, because these TAG stores are turned over and the FA is used for oxidation (5). Increased lipin-1 expression, in addition to facilitating TAG synthesis, also promotes the transcription of key enzymes in FA oxidation (27).

Alternatively, the stored TAGs are hydrolyzed, and together with exogenous FAs they are reesterified for VLDL production. The secretions of TAG and apolipoprotein B are also increased by GC, and insulin antagonizes this action (44–46). Hepatic PAP1 activity is also positively correlated with circulating TAG (5, 13, 47), and the changes in the level of lipin-1 are probably partly responsible for the regulation of VLDL secretion.

We also established that the hepatic responses of PAP1 to GC, glucagon, and insulin are coregulated with those of enzymes involved in controlling gluconeogenesis (5, 9). Transcription of key gluconeogenic enzymes is also regulated through PGC-1α and PPARα. The coregulation of lipin-1 expression and the physical interactions of lipin-1 with PGC-1α and PPARα (27) could help to modulate and integrate gluconeogenesis with the increased capacity for hepatic TAG synthesis and β-oxidation in starvation and diabetes. In this respect, it may be significant that the livers of *fld* mice show a 40% decrease in hepatic glucose production in fasting (48).

In conclusion, GC increases the transcription of the *Lpin1* gene. cAMP synergizes this GC effect in specifically increasing the expression of lipin-1 mRNA in rat and mouse hepatocytes, and there was no significant increase in mRNA for lipin-2 and -3. The effect of dex + CPTcAMP in increasing lipin-1 mRNA production was attenuated by insulin. This control of the transcription of the *Lpin1* gene explains the increased synthesis and expression of lipin-1A and -1B. This in turn accounts for the increased PAP1 activity that is observed after treating hepatocytes with dex + CPTcAMP or in vivo in starvation and diabetes. The FA-stimulated association of lipin-1 with the nucleus probably enables it to regulate transcription and increase FA oxidation in starvation and diabetes. Higher expression of lipin-1 also increases the capacity for hepatic glycerolipid synthesis and secretion in VLDL. This capacity is expressed as FA accumulates and lipin-1 translocates to the ER, where TAGs are produced. This lipin-1-induced increase in TAG synthesis is reflected in vivo in the steatosis that is observed in starvation, diabetes, ischemia, toxic conditions, and ethanol intoxication.

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